Microbiol. Immunol. 158:97 (1992)). It is also one of the few viruses that may integrate its DNA into non-dividing cells. Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate, but space for exogenous DNA is limited to about 4.5 kb. Methods for producing and using such AAVs are known in the art. See, for example, U.S. Patent Nos. 5,139,941, 5,173,414, 5,354,678, 5,436,146, 5,474,935, 5,478,745, and 5,589,377.

[0566] For example, an appropriate AAV vector for use in the present invention will include all the sequences necessary for DNA replication, encapsidation, and host-cell integration. The polynucleotide construct is inserted into the AAV vector using standard cloning methods, such as those found in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989). The recombinant AAV vector is then transfected into packaging cells which are infected with a helper virus, using any standard technique, including lipofection, electroporation, calcium phosphate precipitation, etc. Appropriate helper viruses include adenoviruses, cytomegaloviruses, vaccinia viruses, or herpes viruses. Once the packaging cells are transfected and infected, they will produce infectious AAV viral particles which contain the polynucleotide construct. These viral particles are then used to transduce eukaryotic cells, either *ex vivo* or *in vivo*. The transduced cells will contain the polynucleotide construct integrated into its genome, and will express a fusion protein of the invention.

[0567] Another method of gene therapy involves operably associating heterologous control regions and endogenous polynucleotide sequences (e.g. encoding a polypeptide of the present invention) via homologous recombination (see, e.g., U.S. Patent No. 5,641,670, issued June 24, 1997; International Publication No. WO 96/29411, published September 26, 1996; International Publication No. WO 94/12650, published August 4, 1994; Koller et al., Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); and Zijlstra et al., Nature 342:435-438 (1989), which are herein encorporated by reference. This method involves the activation of a gene which is present in the target cells, but which is not normally expressed in the cells, or is expressed at a lower level than desired.

[0568] Polynucleotide constructs are made, using standard techniques known in the art, which contain the promoter with targeting sequences flanking the promoter. Suitable promoters are described herein. The targeting sequence is sufficiently complementary to an endogenous sequence to permit homologous recombination of the promoter-targeting sequence with the endogenous sequence. The targeting sequence will be sufficiently near the

5' end of the desired endogenous polynucleotide sequence so the promoter will be operably linked to the endogenous sequence upon homologous recombination.

[0569] The promoter and the targeting sequences can be amplified using PCR. Preferably, the amplified promoter contains distinct restriction enzyme sites on the 5' and 3' ends. Preferably, the 3' end of the first targeting sequence contains the same restriction enzyme site as the 5' end of the amplified promoter and the 5' end of the second targeting sequence contains the same restriction site as the 3' end of the amplified promoter. The amplified promoter and targeting sequences are digested and ligated together.

[0570] The promoter-targeting sequence construct is delivered to the cells, either as naked polynucleotide, or in conjunction with transfection-facilitating agents, such as liposomes, viral sequences, viral particles, whole viruses, lipofection, precipitating agents, etc., described in more detail above. The P promoter-targeting sequence can be delivered by any method, included direct needle injection, intravenous injection, topical administration, catheter infusion, particle accelerators, etc. The methods are described in more detail below.

[0571] The promoter-targeting sequence construct is taken up by cells. Homologous recombination between the construct and the endogenous sequence takes place, such that an endogenous sequence is placed under the control of the promoter. The promoter then drives the expression of the endogenous sequence.

[0572] The polynucleotide encoding an albumin fusion protein of the present invention may contain a secretory signal sequence that facilitates secretion of the protein. Typically, the signal sequence is positioned in the coding region of the polynucleotide to be expressed towards or at the 5' end of the coding region. The signal sequence may be homologous or heterologous to the polynucleotide of interest and may be homologous or heterologous to the cells to be transfected. Additionally, the signal sequence may be chemically synthesized using methods known in the art.

[0573] Any mode of administration of any of the above-described polynucleotides constructs can be used so long as the mode results in the expression of one or more molecules in an amount sufficient to provide a therapeutic effect. This includes direct needle injection, systemic injection, catheter infusion, biolistic injectors, particle accelerators (i.e., "gene guns"), gelfoam sponge depots, other commercially available depot materials, osmotic pumps (e.g., Alza minipumps), oral or suppositorial solid (tablet or pill) pharmaceutical formulations, and decanting or topical applications during surgery. For example, direct injection of naked calcium phosphate-precipitated plasmid into rat liver and rat spleen or a

protein-coated plasmid into the portal vein has resulted in gene expression of the foreign gene in the rat livers (Kaneda et al., Science 243:375 (1989)).

[0574] A preferred method of local administration is by direct injection. Preferably, an albumin fusion protein of the present invention complexed with a delivery vehicle is administered by direct injection into or locally within the area of arteries. Administration of a composition locally within the area of arteries refers to injecting the composition centimeters and preferably, millimeters within arteries.

[0575] Another method of local administration is to contact a polynucleotide construct of the present invention in or around a surgical wound. For example, a patient can undergo surgery and the polynucleotide construct can be coated on the surface of tissue inside the wound or the construct can be injected into areas of tissue inside the wound.

[0576] Therapeutic compositions useful in systemic administration, include fusion proteins of the present invention complexed to a targeted delivery vehicle of the present invention. Suitable delivery vehicles for use with systemic administration comprise liposomes comprising ligands for targeting the vehicle to a particular site. In specific embodiments, suitable delivery vehicles for use with systemic administration comprise liposomes comprising albumin fusion proteins of the invention for targeting the vehicle to a particular site.

[0577] Preferred methods of systemic administration, include intravenous injection, aerosol, oral and percutaneous (topical) delivery. Intravenous injections can be performed using methods standard in the art. Aerosol delivery can also be performed using methods standard in the art (see, for example, Stribling et al., Proc. Natl. Acad. Sci. USA 189:11277-11281, 1992, which is incorporated herein by reference). Oral delivery can be performed by complexing a polynucleotide construct of the present invention to a carrier capable of withstanding degradation by digestive enzymes in the gut of an animal. Examples of such carriers, include plastic capsules or tablets, such as those known in the art. Topical delivery can be performed by mixing a polynucleotide construct of the present invention with a lipophilic reagent (e.g., DMSO) that is capable of passing into the skin.

[0578] Determining an effective amount of substance to be delivered can depend upon a number of factors including, for example, the chemical structure and biological activity of the substance, the age and weight of the animal, the precise condition requiring treatment and its severity, and the route of administration. The frequency of treatments depends upon a number of factors, such as the amount of polynucleotide constructs

administered per dose, as well as the health and history of the subject. The precise amount, number of doses, and timing of doses will be determined by the attending physician or veterinarian.

[0579] Albumin fusion proteins of the present invention can be administered to any animal, preferably to mammals and birds. Preferred mammals include humans, dogs, cats, mice, rats, rabbits sheep, cattle, horses and pigs, with humans being particularly preferred.

Biological Activities

[0580] Albumin fusion proteins and/or polynucleotides encoding albumin fusion proteins of the present invention, can be used in assays to test for one or more biological activities. If an albumin fusion protein and/or polynucleotide exhibits an activity in a particular assay, it is likely that the Therapeutic protein corresponding to the fusion portein may be involved in the diseases associated with the biological activity. Thus, the fusion protein could be used to treat the associated disease.

[0581] In preferred embodiments, the present invention encompasses a method of treating a disease or disorder listed in the "Preferred Indication Y" column of Table 1 comprising administering to a patient in which such treatment, prevention or amelioration is desired an albumin fusion protein of the invention that comprises a Therapeutic protein portion corresponding to a Therapeutic protein disclosed in the "Therapeutic Protein X" column of Table 1 (in the same row as the disease or disorder to be treated is listed in the "Preferred Indication Y" column of Table 1) in an amount effective to treat, prevent or ameliorate the disease or disorder.

[0582] In a further preferred embodiment, the present invention encompasses a method of treating a disease or disorder listed for a particular Therapeutic protein in the "Preferred Indication:Y" column of Table 1 comprising administering to a patient in which such treatment, prevention or amelioration is desired an albumin fusion protein of the invention that comprises a Therapeutic protein portion corresponding to the Therapeutic protein for which the indications in the Examples are related in an amount effective to treat, prevent or ameliorate the disease or disorder.

[0583] Specifically contemplated by the present invention are albumin fusion proteins produced by a cell when encoded by the polynucleotides that encode SEQ ID NO:Y. When these polynucleotides are used to express the encoded protein from a cell, the cell's natural

secretion and processing steps produces a protein that lacks the signal sequence explicitly listed in columns 4 and/or 11 of Table 2. The specific amino acid sequence of the listed signal sequence is shown in the specification or is well known in the art. Thus, most preferred embodiments of the present invention include the albumin fusion protein produced by a cell (which would lack the leader sequence shown in columns 4 and/or 11 of Table 2). Also most preferred are polypeptides comprising SEQ ID NO:Y without the specific leader sequence listed in columns 4 and/or 11 of Table 2. Compositions comprising these two preferred embodiments, including pharmaceutical compositions, are also preferred. These albumin fusion proteins are specifically contemplated to treat, prevent, or ameliorate a disease or disorder listed for a particular Therapeutic protein in the "Preferred Indication:Y" column of Table 1.

[0584] In preferred embodiments, fusion proteins of the present invention may be used in the diagnosis, prognosis, prevention and/or treatment of diseases and/or disorders relating to diseases and disorders of the endocrine system (see, for example, "Endocrine Disorders" section below), the nervous system (see, for example, "Neurological Disorders" section below), the immune system (see, for example, "Immune Activity" section below), respiratory system (see, for example, "Respiratory Disorders" section below), cardiovascular system (see, for example, "Cardiovascular Disorders" section below), reproductive system (see, for example, "Reproductive System Disorders" section below) digestive system (see, for example, "Gastrointestinal Disorders" section below), diseases and/or disorders relating to cell proliferation (see, for example, "Hyperproliferative Disorders" section below), and/or diseases or disorders relating to the blood (see, for example, "Blood-Related Disorders" section below).

[0585] In certain embodiments, an albumin fusion protein of the present invention may be used to diagnose and/or prognose diseases and/or disorders associated with the tissue(s) in which the gene corresponding to the Therapeutic protein portion of the fusion protein of the invention is expressed.

[0586] Thus, fusion proteins of the invention and polynucleotides encoding albumin fusion proteins of the invention are useful in the diagnosis, detection and/or treatment of diseases and/or disorders associated with activities that include, but are not limited to, prohormone activation, neurotransmitter activity, cellular signaling, cellular proliferation, cellular differentiation, and cell migration.

[0587] More generally, fusion proteins of the invention and polynucleotides encoding

albumin fusion proteins of the invention may be useful for the diagnosis, prognosis, prevention and/or treatment of diseases and/or disorders associated with the following systems.

Immune Activity

[0588] Albumin fusion proteins of the invention and polynucleotides encoding albumin fusion proteins of the invention may be useful in treating, preventing, diagnosing and/or prognosing diseases, disorders, and/or conditions of the immune system, by, for example, activating or inhibiting the proliferation, differentiation, or mobilization (chemotaxis) of immune cells. Immune cells develop through a process called hematopoiesis, producing myeloid (platelets, red blood cells, neutrophils, and macrophages) and lymphoid (B and T lymphocytes) cells from pluripotent stem cells. The etiology of these immune diseases, disorders, and/or conditions may be genetic, somatic, such as cancer and some autoimmune diseases, acquired (e.g., by chemotherapy or toxins), or infectious. Moreover, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention can be used as a marker or detector of a particular immune system disease or disorder.

[0589] In another embodiment, a fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention, may be used to treat diseases and disorders of the immune system and/or to inhibit or enhance an immune response generated by cells associated with the tissue(s) in which the polypeptide of the invention is expressed.

[0590] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in treating, preventing, diagnosing, and/or prognosing immunodeficiencies, including both congenital and acquired immunodeficiencies. Examples of B cell immunodeficiencies in which immunoglobulin levels B cell function and/or B cell numbers are decreased include: X-linked agammaglobulinemia (Bruton's disease), X-linked infantile agammaglobulinemia, X-linked immunodeficiency with hyper IgM, non X-linked immunodeficiency with hyper IgM, X-linked lymphoproliferative syndrome (XLP), agammaglobulinemia including congenital and acquired agammaglobulinemia, adult onset agammaglobulinemia, late-onset agammaglobulinemia, dysgammaglobulinemia, hypogammaglobulinemia, unspecified hypogammaglobulinemia, recessive agammaglobulinemia (Swiss type), Selective IgM

deficiency, selective IgA deficiency, selective IgG subclass deficiencies, IgG subclass deficiency (with or without IgA deficiency), Ig deficiency with increased IgM, IgG and IgA deficiency with increased IgM, antibody deficiency with normal or elevated Igs, Ig heavy chain deletions, kappa chain deficiency, B cell lymphoproliferative disorder (BLPD), common variable immunodeficiency (CVID), common variable immunodeficiency (CVI) (acquired), and transient hypogammaglobulinemia of infancy.

[0591] In specific embodiments, ataxia-telangiectasia or conditions associated with ataxia-telangiectasia are treated, prevented, diagnosed, and/or prognosing using the, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention.

[0592] Examples of congenital immunodeficiencies in which T cell and/or B cell function and/or number is decreased include, but are not limited to: DiGeorge anomaly, severe combined immunodeficiencies (SCID) (including, but not limited to, X-linked SCID, autosomal recessive SCID, adenosine deaminase deficiency, purine nucleoside phosphorylase (PNP) deficiency, Class II MHC deficiency (Bare lymphocyte syndrome), Wiskott-Aldrich syndrome, and ataxia telangiectasia), thymic hypoplasia, third and fourth pharyngeal pouch syndrome, 22q11.2 deletion, chronic mucocutaneous candidiasis, natural killer cell deficiency (NK), idiopathic CD4+ T-lymphocytopenia, immunodeficiency with predominant T cell defect (unspecified), and unspecified immunodeficiency of cell mediated immunity.

[0593] In specific embodiments, DiGeorge anomaly or conditions associated with DiGeorge anomaly are treated, prevented, diagnosed, and/or prognosed using fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention.

[0594] Other immunodeficiencies that may be treated, prevented, diagnosed, and/or prognosed using fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, include, but are not limited to, chronic granulomatous disease, Chédiak-Higashi syndrome, myeloperoxidase deficiency, leukocyte glucose-6-phosphate dehydrogenase deficiency, X-linked lymphoproliferative syndrome (XLP), leukocyte adhesion deficiency, complement component deficiencies (including C1, C2, C3, C4, C5, C6, C7, C8 and/or C9 deficiencies), reticular dysgenesis, thymic alymphoplasia-aplasia, immunodeficiency with thymoma, severe congenital leukopenia, dysplasia with immunodeficiency, neonatal neutropenia, short limbed dwarfism, and Nezelof syndrome-combined immunodeficiency with Igs.

[0595] In a preferred embodiment, the immunodeficiencies and/or conditions

associated with the immunodeficiencies recited above are treated, prevented, diagnosed and/or prognosed using fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention.

[0596] In a preferred embodiment fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention could be used as an agent to boost immunoresponsiveness among immunodeficient individuals. In specific embodiments, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention could be used as an agent to boost immunoresponsiveness among B cell and/or T cell immunodeficient individuals.

[0597] The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in treating, preventing, diagnosing and/or prognosing autoimmune disorders. Many autoimmune disorders result from inappropriate recognition of self as foreign material by immune cells. This inappropriate recognition results in an immune response leading to the destruction of the host tissue. Therefore, the administration of fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention that can inhibit an immune response, particularly the proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing autoimmune disorders.

[0598] Autoimmune diseases or disorders that may be treated, prevented, diagnosed and/or prognosed by fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to, one or more of the following: systemic lupus erythematosus, rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis, autoimmune thyroiditis, Hashimoto's thyroiditis, autoimmune hemolytic anemia, hemolytic anemia, thrombocytopenia, autoimmune thrombocytopenia purpura, autoimmune neonatal thrombocytopenia, idiopathic thrombocytopenia purpura, purpura (e.g., Henloch-Scoenlein purpura), autoimmunocytopenia, Goodpasture's syndrome, Pemphigus vulgaris, myasthenia gravis, Grave's disease (hyperthyroidism), and insulin-resistant diabetes mellitus.

[0599] Additional disorders that are likely to have an autoimmune component that may be treated, prevented, and/or diagnosed with the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to, type II collagen-induced arthritis, antiphospholipid syndrome, dermatitis, allergic encephalomyelitis, myocarditis, relapsing polychondritis, rheumatic heart disease, neuritis,

uveitis ophthalmia, polyendocrinopathies, Reiter's Disease, Stiff-Man Syndrome, autoimmune pulmonary inflammation, autism, Guillain-Barre Syndrome, insulin dependent diabetes mellitus, and autoimmune inflammatory eye disorders.

[0600] Additional disorders that are likely to have an autoimmune component that may be treated, prevented, diagnosed and/or prognosed with the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to, scleroderma with anti-collagen antibodies (often characterized, e.g., by nucleolar and other nuclear antibodies), mixed connective tissue disease (often characterized, e.g., by antibodies to extractable nuclear antigens (e.g., ribonucleoprotein)), polymyositis (often characterized, e.g., by nonhistone ANA), pernicious anemia (often characterized, e.g., by antiparietal cell, microsomes, and intrinsic factor antibodies), idiopathic Addison's disease (often characterized, e.g., by humoral and cell-mediated adrenal cytotoxicity, infertility (often characterized, e.g., by antispermatozoal antibodies), glomerulonephritis (often characterized, e.g., by glomerular basement membrane antibodies or immune complexes), bullous pemphigoid (often characterized, e.g., by IgG and complement in basement membrane), Sjogren's syndrome (often characterized, e.g., by multiple tissue antibodies, and/or a specific nonhistone ANA (SS-B)), diabetes mellitus (often characterized, e.g., by cell-mediated and humoral islet cell antibodies), and adrenergic drug resistance (including adrenergic drug resistance with asthma or cystic fibrosis) (often characterized, e.g., by beta-adrenergic receptor antibodies).

[0601] Additional disorders that may have an autoimmune component that may be treated, prevented, diagnosed and/or prognosed with the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to, chronic active hepatitis (often characterized, e.g., by smooth muscle antibodies), primary biliary cirrhosis (often characterized, e.g., by mitochondria antibodies), other endocrine gland failure (often characterized, e.g., by specific tissue antibodies in some cases), vitiligo (often characterized, e.g., by melanocyte antibodies), vasculitis (often characterized, e.g., by Ig and complement in vessel walls and/or low serum complement), post-MI (often characterized, e.g., by myocardial antibodies), cardiotomy syndrome (often characterized, e.g., by myocardial antibodies), urticaria (often characterized, e.g., by IgG and IgM antibodies to IgE), atopic dermatitis (often characterized, e.g., by IgG and IgM antibodies to IgE), asthma (often characterized, e.g., by IgG and IgM antibodies to IgE), and many other inflammatory, granulomatous, degenerative, and atrophic disorders.

[0602] In a preferred embodiment, the autoimmune diseases and disorders and/or conditions associated with the diseases and disorders recited above are treated, prevented, diagnosed and/or prognosed using for example, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention. In a specific preferred embodiment, rheumatoid arthritis is treated, prevented, and/or diagnosed using fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention.

[0603] In another specific preferred embodiment, systemic lupus erythematosus is treated, prevented, and/or diagnosed using fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention. In another specific preferred embodiment, idiopathic thrombocytopenia purpura is treated, prevented, and/or diagnosed using fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention.

[0604] In another specific preferred embodiment IgA nephropathy is treated, prevented, and/or diagnosed using fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention.

[0605] In a preferred embodiment, the autoimmune diseases and disorders and/or conditions associated with the diseases and disorders recited above are treated, prevented, diagnosed and/or prognosed using fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention.

[0606] In preferred embodiments, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a immunosuppressive agent(s).

[0607] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in treating, preventing, prognosing, and/or diagnosing diseases, disorders, and/or conditions of hematopoietic cells. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention could be used to increase differentiation and proliferation of hematopoietic cells, including the pluripotent stem cells, in an effort to treat or prevent those diseases, disorders, and/or conditions associated with a decrease in certain (or many) types hematopoietic cells, including but not limited to, leukopenia, neutropenia, anemia, and thrombocytopenia. Alternatively, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention could be used to increase differentiation

and proliferation of hematopoietic cells, including the pluripotent stem cells, in an effort to treat or prevent those diseases, disorders, and/or conditions associated with an increase in certain (or many) types of hematopoietic cells, including but not limited to, histiocytosis.

[0608] Allergic reactions and conditions, such as asthma (particularly allergic asthma) or other respiratory problems, may also be treated, prevented, diagnosed and/or prognosed using fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention. Moreover, these molecules can be used to treat, prevent, prognose, and/or diagnose anaphylaxis, hypersensitivity to an antigenic molecule, or blood group incompatibility.

[0609] Additionally, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, may be used to treat, prevent, diagnose and/or prognose IgE-mediated allergic reactions. Such allergic reactions include, but are not limited to, asthma, rhinitis, and eczema. In specific embodiments, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used to modulate IgE concentrations in vitro or in vivo.

[0610] Moreover, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention have uses in the diagnosis, prognosis, prevention, and/or treatment of inflammatory conditions. For example, since fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may inhibit the activation, proliferation and/or differentiation of cells involved in an inflammatory response, these molecules can be used to prevent and/or treat chronic and acute inflammatory conditions. Such inflammatory conditions include, but are not limited to, for example, inflammation associated with infection (e.g., septic shock, sepsis, or systemic inflammatory response syndrome), ischemia-reperfusion injury, endotoxin lethality, complement-mediated hyperacute rejection, nephritis, cytokine or chemokine induced lung injury, inflammatory bowel disease, Crohn's disease, over production of cytokines (e.g., TNF or IL-1.), respiratory disorders (e.g., asthma and allergy); gastrointestinal disorders (e.g., inflammatory bowel disease); cancers (e.g., gastric, ovarian, lung, bladder, liver, and breast); CNS disorders (e.g., multiple sclerosis; ischemic brain injury and/or stroke, traumatic brain injury, neurodegenerative disorders (e.g., Parkinson's disease and Alzheimer's disease); AIDS-related dementia; and prion disease); cardiovascular disorders (e.g., atherosclerosis, myocarditis, cardiovascular disease, and cardiopulmonary bypass complications); as well as many additional diseases, conditions, and disorders that are characterized by inflammation

(e.g., hepatitis, rheumatoid arthritis, gout, trauma, pancreatitis, sarcoidosis, dermatitis, renal ischemia-reperfusion injury, Grave's disease, systemic lupus erythematosus, diabetes mellitus, and allogenic transplant rejection).

[0611] Because inflammation is a fundamental defense mechanism, inflammatory disorders can effect virtually any tissue of the body. Accordingly, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, have uses in the treatment of tissue-specific inflammatory disorders, including, but not limited to, adrenalitis, alveolitis, angiocholecystitis, appendicitis, balanitis, blepharitis, bronchitis, bursitis, carditis, cellulitis, cervicitis, cholecystitis, chorditis, cochlitis, colitis, conjunctivitis, cystitis, dermatitis, diverticulitis, encephalitis, endocarditis, esophagitis, eustachitis, fibrositis, folliculitis, gastritis, gastroenteritis, gingivitis, glossitis, hepatosplenitis, keratitis, labyrinthitis, laryngitis, lymphangitis, mastitis, media otitis, meningitis, metritis, mucitis, myocarditis, myosititis, myringitis, nephritis, neuritis, orchitis, osteochondritis, otitis, pericarditis, peritendonitis, peritonitis, pharyngitis, phlebitis, poliomyelitis, prostatitis, pulpitis, retinitis, rhinitis, salpingitis, scleritis, sclerochoroiditis, scrotitis, sinusitis, spondylitis, steatitis, stomatitis, synovitis, syringitis, tendonitis, tonsillitis, urethritis, and vaginitis.

[0612] In specific embodiments, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, are useful to diagnose, prognose, prevent, and/or treat organ transplant rejections and graft-versus-host disease. Organ rejection occurs by host immune cell destruction of the transplanted tissue through an immune response. Similarly, an immune response is also involved in GVHD, but, in this case, the foreign transplanted immune cells destroy the host tissues. Polypeptides, antibodies, or polynucleotides of the invention, and/or agonists or antagonists thereof, that inhibit an immune response, particularly the activation, proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing organ rejection or GVHD. In specific embodiments, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, that inhibit an immune response, particularly the activation, proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing experimental allergic and hyperacute xenograft rejection.

[0613] In other embodiments, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, are useful to diagnose, prognose, prevent, and/or treat immune complex diseases, including, but not limited to, serum sickness, post

streptococcal glomerulonephritis, polyarteritis nodosa, and immune complex-induced vasculitis.

[0614] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention can be used to treat, detect, and/or prevent infectious agents. For example, by increasing the immune response, particularly increasing the proliferation activation and/or differentiation of B and/or T cells, infectious diseases may be treated, detected, and/or prevented. The immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may also directly inhibit the infectious agent (refer to section of application listing infectious agents, etc), without necessarily eliciting an immune response.

[0615] In another embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a vaccine adjuvant that enhances immune responsiveness to an antigen. In a specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an adjuvant to enhance tumor-specific immune responses.

[0616] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an adjuvant to enhance anti-viral immune responses. Anti-viral immune responses that may be enhanced using the compositions of the invention as an adjuvant, include virus and virus associated diseases or symptoms described herein or otherwise known in the art. In specific embodiments, the compositions of the invention are used as an adjuvant to enhance an immune response to a virus, disease, or symptom selected from the group consisting of: AIDS, meningitis, Dengue, EBV, and hepatitis (e.g., hepatitis B). In another specific embodiment, the compositions of the invention are used as an adjuvant to enhance an immune response to a virus, disease, or symptom selected from the group consisting of: HIV/AIDS, respiratory syncytial virus, Dengue, rotavirus, Japanese B encephalitis, influenza A and B, parainfluenza, measles, cytomegalovirus, rabies, Junin, Chikungunya, Rift Valley Fever, herpes simplex, and yellow fever.

[0617] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an

adjuvant to enhance anti-bacterial or anti-fungal immune responses. Anti-bacterial or anti-fungal immune responses that may be enhanced using the compositions of the invention as an adjuvant, include bacteria or fungus and bacteria or fungus associated diseases or symptoms described herein or otherwise known in the art. In specific embodiments, the compositions of the invention are used as an adjuvant to enhance an immune response to a bacteria or fungus, disease, or symptom selected from the group consisting of: tetanus, Diphtheria, botulism, and meningitis type B.

[0618] In another specific embodiment, the compositions of the invention are used as an adjuvant to enhance an immune response to a bacteria or fungus, disease, or symptom selected from the group consisting of: *Vibrio cholerae, Mycobacterium leprae, Salmonella typhi, Salmonella paratyphi, Meisseria meningitidis, Streptococcus pneumoniae*, Group B streptococcus, *Shigella spp.*, Enterotoxigenic *Escherichia coli,* Enterohemorrhagic *E. coli*, and *Borrelia burgdorferi.*

[0619] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an adjuvant to enhance anti-parasitic immune responses. Anti-parasitic immune responses that may be enhanced using the compositions of the invention as an adjuvant, include parasite and parasite associated diseases or symptoms described herein or otherwise known in the art. In specific embodiments, the compositions of the invention are used as an adjuvant to enhance an immune response to a parasite. In another specific embodiment, the compositions of the invention are used as an adjuvant to enhance an immune response to Plasmodium (malaria) or Leishmania.

[0620] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may also be employed to treat infectious diseases including silicosis, sarcoidosis, and idiopathic pulmonary fibrosis; for example, by preventing the recruitment and activation of mononuclear phagocytes.

[0621] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an antigen for the generation of antibodies to inhibit or enhance immune mediated responses against polypeptides of the invention.

[0622] In one embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are administered to an

animal (e.g., mouse, rat, rabbit, hamster, guinea pig, pigs, micro-pig, chicken, camel, goat, horse, cow, sheep, dog, cat, non-human primate, and human, most preferably human) to boost the immune system to produce increased quantities of one or more antibodies (e.g., IgG, IgA, IgM, and IgE), to induce higher affinity antibody production and immunoglobulin class switching (e.g., IgG, IgA, IgM, and IgE), and/or to increase an immune response.

[0623] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a stimulator of B cell responsiveness to pathogens.

[0624] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an activator of T cells.

[0625] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an agent that elevates the immune status of an individual prior to their receipt of immunosuppressive therapies.

[0626] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an agent to induce higher affinity antibodies.

[0627] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an agent to increase serum immunoglobulin concentrations.

[0628] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an agent to accelerate recovery of immunocompromised individuals.

[0629] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an agent to boost immunoresponsiveness among aged populations and/or neonates.

[0630] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an immune system enhancer prior to, during, or after bone marrow transplant and/or other transplants (e.g., allogeneic or xenogeneic organ transplantation). With respect to transplantation, compositions of the invention may be administered prior to, concomitant with, and/or after transplantation. In a specific embodiment, compositions of the invention

are administered after transplantation, prior to the beginning of recovery of T-cell populations. In another specific embodiment, compositions of the invention are first administered after transplantation after the beginning of recovery of T cell populations, but prior to full recovery of B cell populations.

[0631] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an agent to boost immunoresponsiveness among individuals having an acquired loss of B cell function. Conditions resulting in an acquired loss of B cell function that may be ameliorated or treated by administering the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, include, but are not limited to, HIV Infection, AIDS, bone marrow transplant, and B cell chronic lymphocytic leukemia (CLL).

[0632] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an agent to boost immunoresponsiveness among individuals having a temporary immune deficiency. Conditions resulting in a temporary immune deficiency that may be ameliorated or treated by administering the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, include, but are not limited to, recovery from viral infections (e.g., influenza), conditions associated with malnutrition, recovery from infectious mononucleosis, or conditions associated with stress, recovery from measles, recovery from blood transfusion, and recovery from surgery.

[0633] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a regulator of antigen presentation by monocytes, dendritic cells, and/or B-cells. In one embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention enhance antigen presentation or antagonize antigen presentation in vitro or in vivo. Moreover, in related embodiments, this enhancement or antagonism of antigen presentation may be useful as an anti-tumor treatment or to modulate the immune system.

[0634] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as an agent to direct an individual's immune system towards development of a humoral response (i.e. TH2) as opposed to a TH1 cellular response.

[0635] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a means to induce tumor proliferation and thus make it more susceptible to anti-neoplastic agents. For example, multiple myeloma is a slowly dividing disease and is thus refractory to virtually all anti-neoplastic regimens. If these cells were forced to proliferate more rapidly their susceptibility profile would likely change.

[0636] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a stimulator of B cell production in pathologies such as AIDS, chronic lymphocyte disorder and/or Common Variable Immunodificiency.

[0637] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a therapy for generation and/or regeneration of lymphoid tissues following surgery, trauma or genetic defect. In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used in the pretreatment of bone marrow samples prior to transplant.

[0638] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a gene-based therapy for genetically inherited disorders resulting in immuno-incompetence/immunodeficiency such as observed among SCID patients.

[0639] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a means of activating monocytes/macrophages to defend against parasitic diseases that effect monocytes such as Leishmania.

[0640] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a means of regulating secreted cytokines that are elicited by polypeptides of the invention.

[0641] In another embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used in one or more of the applications decribed herein, as they may apply to veterinary medicine.

[0642] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a means of blocking various aspects of immune responses to foreign agents or self. Examples of

diseases or conditions in which blocking of certain aspects of immune responses may be desired include autoimmune disorders such as lupus, and arthritis, as well as immunoresponsiveness to skin allergies, inflammation, bowel disease, injury and diseases/disorders associated with pathogens.

[0643] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a therapy for preventing the B cell proliferation and Ig secretion associated with autoimmune diseases such as idiopathic thrombocytopenic purpura, systemic lupus erythematosus and multiple sclerosis.

[0644] In another specific embodiment, polypeptides, antibodies, polynucleotides and/or agonists or antagonists of the present fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention invention are used as a inhibitor of B and/or T cell migration in endothelial cells. This activity disrupts tissue architecture or cognate responses and is useful, for example in disrupting immune responses, and blocking sepsis.

[0645] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a therapy for chronic hypergammaglobulinemia evident in such diseases as monoclonal gammopathy of undetermined significance (MGUS), Waldenstrom's disease, related idiopathic monoclonal gammopathies, and plasmacytomas.

[0646] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be employed for instance to inhibit polypeptide chemotaxis and activation of macrophages and their precursors, and of neutrophils, basophils, B lymphocytes and some T-cell subsets, e.g., activated and CD8 cytotoxic T cells and natural killer cells, in certain autoimmune and chronic inflammatory and infective diseases. Examples of autoimmune diseases are described herein and include multiple sclerosis, and insulin-dependent diabetes.

[0647] The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may also be employed to treat idiopathic hyper-eosinophilic syndrome by, for example, preventing eosinophil production and migration.

[0648] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to enhance or inhibit complement mediated cell lysis.

[0649] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to enhance or inhibit antibody dependent cellular cytotoxicity.

[0650] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may also be employed for treating atherosclerosis, for example, by preventing monocyte infiltration in the artery wall.

[0651] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be employed to treat adult respiratory distress syndrome (ARDS).

[0652] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful for stimulating wound and tissue repair, stimulating angiogenesis, and/or stimulating the repair of vascular or lymphatic diseases or disorders. Additionally, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used to stimulate the regeneration of mucosal surfaces.

[0653] In a specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to diagnose, prognose, treat, and/or prevent a disorder characterized by primary or acquired immunodeficiency, deficient serum immunoglobulin production, recurrent infections, and/or immune system dysfunction. Moreover, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used to treat or prevent infections of the joints, bones, skin, and/or parotid glands, blood-borne infections (e.g., sepsis, meningitis, septic arthritis, and/or osteomyelitis), autoimmune diseases (e.g., those disclosed herein), inflammatory disorders, and malignancies, and/or any disease or disorder or condition associated with these infections, diseases, disorders and/or malignancies) including, but not limited to, CVID, other primary immune deficiencies, HIV disease, CLL, recurrent bronchitis, sinusitis, otitis media, conjunctivitis, pneumonia, hepatitis, meningitis, herpes zoster (e.g., severe herpes zoster), and/or pneumocystis carnii. Other diseases and disorders that may be prevented, diagnosed, prognosed, and/or treated with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to, HIV infection, HTLV-BLV infection, lymphopenia, phagocyte bactericidal dysfunction anemia, thrombocytopenia, and

hemoglobinuria.

[0654] In another embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to treat, and/or diagnose an individual having common variable immunodeficiency disease ("CVID"; also known as "acquired agammaglobulinemia" and "acquired hypogammaglobulinemia") or a subset of this disease.

[0655] In a specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used to diagnose, prognose, prevent, and/or treat cancers or neoplasms including immune cell or immune tissue-related cancers or neoplasms. Examples of cancers or neoplasms that may be prevented, diagnosed, or treated by fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to, acute myelogenous leukemia, chronic myelogenous leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, acute lymphocytic anemia (ALL) Chronic lymphocyte leukemia, plasmacytomas, multiple myeloma, Burkitt's lymphoma, EBV-transformed diseases, and/or diseases and disorders described in the section entitled "Hyperproliferative Disorders" elsewhere herein.

[0656] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a therapy for decreasing cellular proliferation of Large B-cell Lymphomas.

[0657] In another specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used as a means of decreasing the involvement of B cells and Ig associated with Chronic Myelogenous Leukemia.

[0658] In specific embodiments, the compositions of the invention are used as an agent to boost immunoresponsiveness among B cell immunodeficient individuals, such as, for example, an individual who has undergone a partial or complete splenectomy.

Blood-Related Disorders

[0659] The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used to modulate hemostatic (the stopping of bleeding) or thrombolytic (clot dissolving) activity. For example, by increasing hemostatic or thrombolytic activity, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention could be used to treat or prevent blood coagulation

diseases, disorders, and/or conditions (e.g., afibrinogenemia, factor deficiencies, hemophilia), blood platelet diseases, disorders, and/or conditions (e.g., thrombocytopenia), or wounds resulting from trauma, surgery, or other causes. Alternatively, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention that can decrease hemostatic or thrombolytic activity could be used to inhibit or dissolve clotting. These molecules could be important in the treatment or prevention of heart attacks (infarction), strokes, or scarring.

[0660] In specific embodiments, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used to prevent, diagnose, prognose, and/or treat thrombosis, arterial thrombosis, venous thrombosis, thromboembolism, pulmonary embolism, atherosclerosis, myocardial infarction, transient ischemic attack, unstable angina. In specific embodiments, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used for the prevention of occulsion of saphenous grafts, for reducing the risk of periprocedural thrombosis as might accompany angioplasty procedures, for reducing the risk of stroke in patients with atrial fibrillation including nonrheumatic atrial fibrillation, for reducing the risk of embolism associated with mechanical heart valves and or mitral valves disease. Other uses for the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, include, but are not limited to, the prevention of occlusions in extrcorporeal devices (e.g., intravascular canulas, vascular access shunts in hemodialysis patients, hemodialysis machines, and cardiopulmonary bypass machines).

[0661] In another embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, may be used to prevent, diagnose, prognose, and/or treat diseases and disorders of the blood and/or blood forming organs associated with the tissue(s) in which the polypeptide of the invention is expressed.

[0662] The fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used to modulate hematopoietic activity (the formation of blood cells). For example, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used to increase the quantity of all or subsets of blood cells, such as, for example, erythrocytes, lymphocytes (B or T cells), myeloid cells (e.g., basophils, eosinophils, neutrophils, mast cells, macrophages) and platelets. The ability to decrease the quantity of blood cells or subsets of

blood cells may be useful in the prevention, detection, diagnosis and/or treatment of anemias and leukopenias described below. Alternatively, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used to decrease the quantity of all or subsets of blood cells, such as, for example, erythrocytes, lymphocytes (B or T cells), myeloid cells (e.g., basophils, eosinophils, neutrophils, mast cells, macrophages) and platelets.. The ability to decrease the quantity of blood cells or subsets of blood cells may be useful in the prevention, detection, diagnosis and/or treatment of leukocytoses, such as, for example eosinophilia.

[0663] The fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used to prevent, treat, or diagnose blood dyscrasia.

[0664] Anemias are conditions in which the number of red blood cells or amount of hemoglobin (the protein that carries oxygen) in them is below normal. Anemia may be caused by excessive bleeding, decreased red blood cell production, or increased red blood cell destruction (hemolysis). The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in treating, preventing, and/or diagnosing anemias. Anemias that may be treated prevented or diagnosed by the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include iron deficiency anemia, hypochromic anemia, microcytic anemia, chlorosis, hereditary siderob;astic anemia, idiopathic acquired sideroblastic anemia, red cell aplasia, megaloblastic anemia (e.g., pernicious anemia, (vitamin B12 deficiency) and folic acid deficiency anemia), aplastic anemia, hemolytic anemias (e.g., autoimmune helolytic anemia, microangiopathic hemolytic anemia, and paroxysmal nocturnal hemoglobinuria). The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in treating, preventing, and/or diagnosing anemias associated with diseases including but not limited to, anemias associated with systemic lupus erythematosus, cancers, lymphomas, chronic renal disease, and enlarged spleens. The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in treating, preventing, and/or diagnosing anemias arising from drug treatments such as anemias associated with methyldopa, dapsone, and/or sulfadrugs. Additionally, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in treating, preventing, and/or diagnosing anemias associated with abnormal red blood cell architecture including but not limited to, hereditary spherocytosis, hereditary elliptocytosis, glucose-6-phosphate

dehydrogenase deficiency, and sickle cell anemia.

[0665] The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in treating, preventing, and/or diagnosing hemoglobin abnormalities, (e.g., those associated with sickle cell anemia, hemoglobin C disease, hemoglobin S-C disease, and hemoglobin E disease). Additionally, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in diagnosing, prognosing, preventing, and/or treating thalassemias, including, but not limited to, major and minor forms of alpha-thalassemia and beta-thalassemia.

[0666] In another embodiment, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in diagnosing, prognosing, preventing, and/or treating bleeding disorders including, but not limited to, thrombocytopenia (e.g., idiopathic thrombocytopenic purpura, and thrombotic thrombocytopenic purpura), Von Willebrand's disease, hereditary platelet disorders (e.g., storage pool disease such as Chediak-Higashi and Hermansky-Pudlak syndromes, thromboxane A2 dysfunction, thromboasthenia, and Bernard-Soulier syndrome), hemolytic-uremic syndrome, hemophelias such as hemophelia A or Factor VII deficiency and Christmas disease or Factor IX deficiency, Hereditary Hemorhhagic Telangiectsia, also known as Rendu-Osler-Weber syndrome, allergic purpura (Henoch Schonlein purpura) and disseminated intravascular coagulation.

[0667] The effect of the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention on the clotting time of blood may be monitored using any of the clotting tests known in the art including, but not limited to, whole blood partial thromboplastin time (PTT), the activated partial thromboplastin time (aPTT), the activated clotting time (ACT), the recalcified activated clotting time, or the Lee-White Clotting time.

[0668] Several diseases and a variety of drugs can cause platelet dysfunction. Thus, in a specific embodiment, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in diagnosing, prognosing, preventing, and/or treating acquired platelet dysfunction such as platelet dysfunction accompanying kidney failure, leukemia, multiple myeloma, cirrhosis of the liver, and systemic lupus erythematosus as well as platelet dysfunction associated with drug treatments, including treatment with aspirin, ticlopidine, nonsteroidal anti-inflammatory drugs (used for

arthritis, pain, and sprains), and penicillin in high doses.

[0669] In another embodiment, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in diagnosing, prognosing, preventing, and/or treating diseases and disorders characterized by or associated with increased or decreased numbers of white blood cells. Leukopenia occurs when the number of white blood cells decreases below normal. Leukopenias include, but are not limited to, neutropenia and lymphocytopenia. An increase in the number of white blood cells compared to normal is known as leukocytosis. The body generates increased numbers of white blood cells during infection. Thus, leukocytosis may simply be a normal physiological parameter that reflects infection. Alternatively, leukocytosis may be an indicator of injury or other disease such as cancer. Leokocytoses, include but are not limited to, eosinophilia, and accumulations of macrophages. In specific embodiments, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in diagnosing, prognosing, preventing, and/or treating leukopenia. In other specific embodiments, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in diagnosing, prognosing, preventing, and/or treating leukocytosis.

[0670] Leukopenia may be a generalized decreased in all types of white blood cells, or may be a specific depletion of particular types of white blood cells. Thus, in specific embodiments, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in diagnosing, prognosing, preventing, and/or treating decreases in neutrophil numbers, known as neutropenia. Neutropenias that may be diagnosed, prognosed, prevented, and/or treated by the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to, infantile genetic agranulocytosis, familial neutropenia, cyclic neutropenia, neutropenias resulting from or associated with dietary deficiencies (e.g., vitamin B 12 deficiency or folic acid deficiency), neutropenias resulting from or associated with drug treatments (e.g., antibiotic regimens such as penicillin treatment, sulfonamide treatment, anticoagulant treatment, anticonvulsant drugs, anti-thyroid drugs, and cancer chemotherapy), and neutropenias resulting from increased neutrophil destruction that may occur in association with some bacterial or viral infections, allergic disorders, autoimmune diseases, conditions in which an individual has an enlarged spleen (e.g., Felty syndrome, malaria and sarcoidosis), and some drug treatment regimens.

[0671] The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in diagnosing, prognosing, preventing, and/or treating lymphocytopenias (decreased numbers of B and/or T lymphocytes), including, but not limited to, lymphocytopenias resulting from or associated with stress, drug treatments (e.g., drug treatment with corticosteroids, cancer chemotherapies, and/or radiation therapies), AIDS infection and/or other diseases such as, for example, cancer, rheumatoid arthritis, systemic lupus erythematosus, chronic infections, some viral infections and/or hereditary disorders (e.g., DiGeorge syndrome, Wiskott-Aldrich Syndome, severe combined immunodeficiency, ataxia telangiectsia).

[0672] The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in diagnosing, prognosing, preventing, and/or treating diseases and disorders associated with macrophage numbers and/or macrophage function including, but not limited to, Gaucher's disease, Niemann-Pick disease, Letterer-Siwe disease and Hand-Schuller-Christian disease.

[0673] In another embodiment, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in diagnosing, prognosing, preventing, and/or treating diseases and disorders associated with eosinophil numbers and/or eosinophil function including, but not limited to, idiopathic hypereosinophilic syndrome, eosinophilia-myalgia syndrome, and Hand-Schuller-Christian disease.

[0674] In yet another embodiment, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in diagnosing, prognosing, preventing, and/or treating leukemias and lymphomas including, but not limited to, acute lymphocytic (lymphpblastic) leukemia (ALL), acute myeloid (myelocytic, myelogenous, myeloblastic, or myelomonocytic) leukemia, chronic lymphocytic leukemia (e.g., B cell leukemias, T cell leukemias, Sezary syndrome, and Hairy cell leukenia), chronic myelocytic (myeloid, myelogenous, or granulocytic) leukemia, Hodgkin's lymphoma, non-hodgkin's lymphoma, Burkitt's lymphoma, and mycosis fungoides.

[0675] In other embodiments, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in diagnosing, prognosing, preventing, and/or treating diseases and disorders of plasma cells including, but not limited to, plasma cell dyscrasias, monoclonal gammaopathies, monoclonal gammopathies of undetermined significance, multiple myeloma, macroglobulinemia,

Waldenstrom's macroglobulinemia, cryoglobulinemia, and Raynaud's phenomenon.

[0676] In other embodiments, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in treating, preventing, and/or diagnosing myeloproliferative disorders, including but not limited to, polycythemia vera, relative polycythemia, secondary polycythemia, myelofibrosis, acute myelofibrosis, agnogenic myelod metaplasia, thrombocythemia, (including both primary and seconday thrombocythemia) and chronic myelocytic leukemia.

[0677] In other embodiments, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful as a treatment prior to surgery, to increase blood cell production.

[0678] In other embodiments, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful as an agent to enhance the migration, phagocytosis, superoxide production, antibody dependent cellular cytotoxicity of neutrophils, eosionophils and macrophages.

[0679] In other embodiments, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful as an agent to increase the number of stem cells in circulation prior to stem cells pheresis. In another specific embodiment, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful as an agent to increase the number of stem cells in circulation prior to platelet pheresis.

[0680] In other embodiments, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful as an agent to increase cytokine production.

[0681] In other embodiments, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in preventing, diagnosing, and/or treating primary hematopoietic disorders.

Hyperproliferative Disorders

[0682] In certain embodiments, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention can be used to treat or detect hyperproliferative disorders, including neoplasms. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may

inhibit the proliferation of the disorder through direct or indirect interactions. Alternatively, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may proliferate other cells which can inhibit the hyperproliferative disorder.

[0683] For example, by increasing an immune response, particularly increasing antigenic qualities of the hyperproliferative disorder or by proliferating, differentiating, or mobilizing T-cells, hyperproliferative disorders can be treated. This immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, decreasing an immune response may also be a method of treating hyperproliferative disorders, such as a chemotherapeutic agent.

[0684] Examples of hyperproliferative disorders that can be treated or detected by fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to neoplasms located in the: colon, abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous (central and peripheral), lymphatic system, pelvis, skin, soft tissue, spleen, thorax, and urogenital tract.

[0685] Similarly, other hyperproliferative disorders can also be treated or detected by fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention. Examples of such hyperproliferative disorders include, but are not limited to: Acute Childhood Lymphoblastic Leukemia, Acute Lymphoblastic Leukemia, Acute Lymphocytic Leukemia, Acute Myeloid Leukemia, Adrenocortical Carcinoma, Adult (Primary) Hepatocellular Cancer, Adult (Primary) Liver Cancer, Adult Acute Lymphocytic Leukemia, Adult Acute Myeloid Leukemia, Adult Hodgkin's Disease, Adult Hodgkin's Lymphoma, Adult Lymphocytic Leukemia, Adult Non-Hodgkin's Lymphoma, Adult Primary Liver Cancer, Adult Soft Tissue Sarcoma, AIDS-Related Lymphoma, AIDS-Related Malignancies, Anal Cancer, Astrocytoma, Bile Duct Cancer, Bladder Cancer, Bone Cancer, Brain Stem Glioma, Brain Tumors, Breast Cancer, Cancer of the Renal Pelvis and Ureter, Central Nervous System (Primary) Lymphoma, Central Nervous System Lymphoma, Cerebellar Astrocytoma, Cerebral Astrocytoma, Cervical Cancer, Childhood (Primary) Hepatocellular Cancer, Childhood (Primary) Liver Cancer, Childhood Acute Lymphoblastic Leukemia, Childhood Acute Myeloid Leukemia, Childhood Brain Stem Glioma, Childhood Cerebellar Astrocytoma, Childhood Cerebral Astrocytoma, Childhood Extracranial Germ Cell Tumors, Childhood Hodgkin's Disease, Childhood Hodgkin's Lymphoma, Childhood Hypothalamic and Visual Pathway Glioma, Childhood Lymphoblastic Leukemia, Childhood

Medulloblastoma, Childhood Non-Hodgkin's Lymphoma, Childhood Pineal and Supratentorial Primitive Neuroectodermal Tumors, Childhood Primary Liver Cancer, Childhood Rhabdomyosarcoma, Childhood Soft Tissue Sarcoma, Childhood Visual Pathway and Hypothalamic Glioma, Chronic Lymphocytic Leukemia, Chronic Myelogenous Leukemia, Colon Cancer, Cutaneous T-Cell Lymphoma, Endocrine Pancreas Islet Cell Carcinoma, Endometrial Cancer, Ependymoma, Epithelial Cancer, Esophageal Cancer, Ewing's Sarcoma and Related Tumors, Exocrine Pancreatic Cancer, Extracranial Germ Cell Tumor, Extragonadal Germ Cell Tumor, Extrahepatic Bile Duct Cancer, Eye Cancer, Female Breast Cancer, Gaucher's Disease, Gallbladder Cancer, Gastric Cancer, Gastrointestinal Carcinoid Tumor, Gastrointestinal Tumors, Germ Cell Tumors, Gestational Trophoblastic Tumor, Hairy Cell Leukemia, Head and Neck Cancer, Hepatocellular Cancer, Hodgkin's Disease, Hodgkin's Lymphoma, Hypergammaglobulinemia, Hypopharyngeal Cancer, Intestinal Cancers, Intraocular Melanoma, Islet Cell Carcinoma, Islet Cell Pancreatic Cancer, Kaposi's Sarcoma, Kidney Cancer, Laryngeal Cancer, Lip and Oral Cavity Cancer, Liver Cancer, Lung Cancer, Lymphoproliferative Disorders, Macroglobulinemia, Male Breast Cancer, Malignant Mesothelioma, Malignant Thymoma, Medulloblastoma, Melanoma, Mesothelioma, Metastatic Occult Primary Squamous Neck Cancer, Metastatic Primary Squamous Neck Cancer, Metastatic Squamous Neck Cancer, Multiple Myeloma, Multiple Myeloma/Plasma Cell Neoplasm, Myelodysplastic Syndrome, Myelogenous Leukemia, Myeloid Leukemia, Myeloproliferative Disorders, Nasal Cavity and Paranasal Sinus Cancer, Nasopharyngeal Cancer, Neuroblastoma, Non-Hodgkin's Lymphoma During Pregnancy, Nonmelanoma Skin Cancer, Non-Small Cell Lung Cancer, Occult Primary Metastatic Squamous Neck Cancer, Oropharyngeal Cancer, Osteo-/Malignant Fibrous Sarcoma, Osteosarcoma/Malignant Fibrous Histiocytoma, Osteosarcoma/Malignant Fibrous Histiocytoma of Bone, Ovarian Epithelial Cancer, Ovarian Germ Cell Tumor, Ovarian Low Malignant Potential Tumor, Pancreatic Cancer, Paraproteinemias, Purpura, Parathyroid Cancer, Penile Cancer, Pheochromocytoma, Pituitary Tumor, Plasma Cell Neoplasm/Multiple Myeloma, Primary Central Nervous System Lymphoma, Primary Liver Cancer, Prostate Cancer, Rectal Cancer, Renal Cell Cancer, Renal Pelvis and Ureter Cancer, Retinoblastoma, Rhabdomyosarcoma, Salivary Gland Cancer, Sarcoidosis Sarcomas, Sezary Syndrome, Skin Cancer, Small Cell Lung Cancer, Small Intestine Cancer, Soft Tissue Sarcoma, Squamous Neck Cancer, Stomach Cancer, Supratentorial Primitive Neuroectodermal and Pineal Tumors, T-Cell Lymphoma, Testicular Cancer, Thymoma,

Thyroid Cancer, Transitional Cell Cancer of the Renal Pelvis and Ureter, Transitional Renal Pelvis and Ureter Cancer, Trophoblastic Tumors, Ureter and Renal Pelvis Cell Cancer, Urethral Cancer, Uterine Cancer, Uterine Sarcoma, Vaginal Cancer, Visual Pathway and Hypothalamic Glioma, Vulvar Cancer, Waldenstrom's Macroglobulinemia, Wilms' Tumor, and any other hyperproliferative disease, besides neoplasia, located in an organ system listed above.

[0686] In another preferred embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to diagnose, prognose, prevent, and/or treat premalignant conditions and to prevent progression to a neoplastic or malignant state, including but not limited to those disorders described above. Such uses are indicated in conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred (for review of such abnormal growth conditions, see Robbins and Angell, 1976, Basic Pathology, 2d Ed., W. B. Saunders Co., Philadelphia, pp. 68-79.)

[0687] Hyperplasia is a form of controlled cell proliferation, involving an increase in cell number in a tissue or organ, without significant alteration in structure or function. Hyperplastic disorders which can be diagnosed, prognosed, prevented, and/or treated with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to, angiofollicular mediastinal lymph node hyperplasia, angiolymphoid hyperplasia with eosinophilia, atypical melanocytic hyperplasia, basal cell hyperplasia, benign giant lymph node hyperplasia, cementum hyperplasia, congenital adrenal hyperplasia, congenital sebaceous hyperplasia, cystic hyperplasia, cystic hyperplasia of the breast, denture hyperplasia, ductal hyperplasia, endometrial hyperplasia, fibromuscular hyperplasia, focal epithelial hyperplasia, gingival hyperplasia, inflammatory fibrous hyperplasia, inflammatory papillary hyperplasia, intravascular papillary endothelial hyperplasia, nodular hyperplasia of prostate, nodular regenerative hyperplasia, pseudoepitheliomatous hyperplasia, senile sebaceous hyperplasia, and verrucous hyperplasia.

[0688] Metaplasia is a form of controlled cell growth in which one type of adult or fully differentiated cell substitutes for another type of adult cell. Metaplastic disorders which can be diagnosed, prognosed, prevented, and/or treated with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not

limited to, agnogenic myeloid metaplasia, apocrine metaplasia, atypical metaplasia, autoparenchymatous metaplasia, connective tissue metaplasia, epithelial metaplasia, intestinal metaplasia, metaplastic anemia, metaplastic ossification, metaplastic polyps, myeloid metaplasia, primary myeloid metaplasia, secondary myeloid metaplasia, squamous metaplasia, squamous metaplasia of amnion, and symptomatic myeloid metaplasia.

[0689] Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia; it is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply stained nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs where there exists chronic irritation or inflammation. Dysplastic disorders which can be diagnosed, prognosed, prevented, and/or treated with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to, anhidrotic ectodermal dysplasia, anterofacial dysplasia, asphyxiating thoracic dysplasia, atriodigital dysplasia, bronchopulmonary dysplasia, cerebral dysplasia, cervical dysplasia, chondroectodermal dysplasia, cleidocranial dysplasia, congenital ectodermal dysplasia, craniodiaphysial dysplasia, craniocarpotarsal dysplasia, craniometaphysial dysplasia, dentin dysplasia, diaphysial dysplasia, ectodermal dysplasia, enamel dysplasia, encephalo-ophthalmic dysplasia, dysplasia epiphysialis hemimelia, dysplasia epiphysialis multiplex, dysplasia epiphysialis punctata, epithelial dysplasia, faciodigitogenital dysplasia, familial fibrous dysplasia of jaws, familial white folded dysplasia, fibromuscular dysplasia, fibrous dysplasia of bone, florid osseous dysplasia, hereditary renal-retinal dysplasia, hidrotic ectodermal dysplasia, hypohidrotic ectodermal dysplasia, lymphopenic thymic dysplasia, mammary dysplasia, mandibulofacial dysplasia, metaphysial dysplasia, Mondini dysplasia, monostotic fibrous dysplasia, mucoepithelial dysplasia, multiple epiphysial dysplasia, oculoauriculovertebral dysplasia, oculodentodigital dysplasia, oculovertebral dysplasia, odontogenic dysplasia, ophthalmomandibulomelic dysplasia, periapical cemental dysplasia, polyostotic fibrous dysplasia, pseudoachondroplastic spondyloepiphysial dysplasia, retinal dysplasia, septo-optic dysplasia, spondyloepiphysial dysplasia, and ventriculoradial dysplasia.

[0690] Additional pre-neoplastic disorders which can be diagnosed, prognosed, prevented, and/or treated with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to, benign dysproliferative disorders (e.g., benign tumors, fibrocystic conditions, tissue hypertrophy,

intestinal polyps, colon polyps, and esophageal dysplasia), leukoplakia, keratoses, Bowen's disease, Farmer's Skin, solar cheilitis, and solar keratosis.

[0691] In another embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, may be used to diagnose and/or prognose disorders associated with the tissue(s) in which the polypeptide of the invention is expressed.

[0692] In another embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention conjugated to a toxin or a radioactive isotope, as described herein, may be used to treat cancers and neoplasms, including, but not limited to, those described herein. In a further preferred embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention conjugated to a toxin or a radioactive isotope, as described herein, may be used to treat acute myelogenous leukemia.

[0693] Additionally, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may affect apoptosis, and therefore, would be useful in treating a number of diseases associated with increased cell survival or the inhibition of apoptosis. For example, diseases associated with increased cell survival or the inhibition of apoptosis that could be diagnosed, prognosed, prevented, and/or treated by polynucleotides, polypeptides, and/or agonists or antagonists of the invention, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, including, but not limited to colon cancer, cardiac tumors, pancreatic cancer, melanoma, retinoblastoma, glioblastoma, lung cancer, intestinal cancer, testicular cancer, stomach cancer, neuroblastoma, myxoma, myoma, lymphoma, endothelioma, osteoblastoma, osteoclastoma, osteosarcoma, chondrosarcoma, adenoma, breast cancer, prostate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders such as, multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis and rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), inflammation, graft v. host disease, acute graft rejection, and chronic graft rejection.

[0694] In preferred embodiments, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to inhibit growth, progression, and/or metastasis of cancers, in particular those listed above.

[0695] Additional diseases or conditions associated with increased cell survival that could be diagnosed, prognosed, prevented, and/or treated by fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as leukemia (including acute leukemias (e.g., acute lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (e.g., chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia)), polycythemia vera, lymphomas (e.g., Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, emangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma, and retinoblastoma.

[0696] Diseases associated with increased apoptosis that could be diagnosed, prognosed, prevented, and/or treated by fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, include AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, retinitis pigmentosa, cerebellar degeneration and brain tumor or prior associated disease); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis and rheumatoid arthritis) myelodysplastic syndromes (such as aplastic anemia), graft v. host disease, ischemic injury (such as that caused by myocardial infarction, stroke and reperfusion injury), liver injury (e.g., hepatitis related liver injury, ischemia/reperfusion injury, cholestosis (bile duct

injury) and liver cancer); toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia and anorexia.

[0697] Hyperproliferative diseases and/or disorders that could be diagnosed, prognosed, prevented, and/or treated by fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, include, but are not limited to, neoplasms located in the liver, abdomen, bone, breast, digestive system, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous system (central and peripheral), lymphatic system, pelvis, skin, soft tissue, spleen, thorax, and urogenital tract.

[0698] Similarly, other hyperproliferative disorders can also be diagnosed, prognosed, prevented, and/or treated by fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention. Examples of such hyperproliferative disorders include, but are not limited to: hypergammaglobulinemia, lymphoproliferative disorders, paraproteinemias, purpura, sarcoidosis, Sezary Syndrome, Waldenstron's macroglobulinemia, Gaucher's Disease, histiocytosis, and any other hyperproliferative disease, besides neoplasia, located in an organ system listed above.

[0699] Another preferred embodiment utilizes polynucleotides encoding albumin fusion proteins of the invention to inhibit aberrant cellular division, by gene therapy using the present invention, and/or protein fusions or fragments thereof.

[0700] Thus, the present invention provides a method for treating cell proliferative disorders by inserting into an abnormally proliferating cell a polynucleotide encoding an albumin fusion protein of the present invention, wherein said polynucleotide represses said expression.

[0701] Another embodiment of the present invention provides a method of treating cell-proliferative disorders in individuals comprising administration of one or more active gene copies of the present invention to an abnormally proliferating cell or cells. In a preferred embodiment, polynucleotides of the present invention is a DNA construct comprising a recombinant expression vector effective in expressing a DNA sequence encoding said polynucleotides. In another preferred embodiment of the present invention, the DNA construct encoding the fusion protein of the present invention is inserted into cells to be treated utilizing a retrovirus, or more preferably an adenoviral vector (See G J. Nabel, et. al., PNAS 1999 96: 324-326, which is hereby incorporated by reference). In a most preferred embodiment, the viral vector is defective and will not transform non-proliferating cells, only

proliferating cells. Moreover, in a preferred embodiment, the polynucleotides of the present invention inserted into proliferating cells either alone, or in combination with or fused to other polynucleotides, can then be modulated via an external stimulus (i.e. magnetic, specific small molecule, chemical, or drug administration, etc.), which acts upon the promoter upstream of said polynucleotides to induce expression of the encoded protein product. As such the beneficial therapeutic affect of the present invention may be expressly modulated (i.e. to increase, decrease, or inhibit expression of the present invention) based upon said external stimulus.

[0702] Polynucleotides of the present invention may be useful in repressing expression of oncogenic genes or antigens. By "repressing expression of the oncogenic genes " is intended the suppression of the transcription of the gene, the degradation of the gene transcript (pre-message RNA), the inhibition of splicing, the destruction of the messenger RNA, the prevention of the post-translational modifications of the protein, the destruction of the protein, or the inhibition of the normal function of the protein.

[0703] For local administration to abnormally proliferating cells, polynucleotides of the present invention may be administered by any method known to those of skill in the art including, but not limited to transfection, electroporation, microinjection of cells, or in vehicles such as liposomes, lipofectin, or as naked polynucleotides, or any other method described throughout the specification. The polynucleotide of the present invention may be delivered by known gene delivery systems such as, but not limited to, retroviral vectors (Gilboa, J. Virology 44:845 (1982); Hocke, Nature 320:275 (1986); Wilson, et al., Proc. Natl. Acad. Sci. U.S.A. 85:3014), vaccinia virus system (Chakrabarty et al., Mol. Cell Biol. 5:3403 (1985) or other efficient DNA delivery systems (Yates et al., Nature 313:812 (1985)) known to those skilled in the art. These references are exemplary only and are hereby incorporated by reference. In order to specifically deliver or transfect cells which are abnormally proliferating and spare non-dividing cells, it is preferable to utilize a retrovirus, or adenoviral (as described in the art and elsewhere herein) delivery system known to those of skill in the art. Since host DNA replication is required for retroviral DNA to integrate and the retrovirus will be unable to self replicate due to the lack of the retrovirus genes needed for its life cycle. Utilizing such a retroviral delivery system for polynucleotides of the present invention will target said gene and constructs to abnormally proliferating cells and will spare the non-dividing normal cells.

[0704] The polynucleotides of the present invention may be delivered directly to cell

proliferative disorder/disease sites in internal organs, body cavities and the like by use of imaging devices used to guide an injecting needle directly to the disease site. The polynucleotides of the present invention may also be administered to disease sites at the time of surgical intervention.

[0705] By "cell proliferative disease" is meant any human or animal disease or disorder, affecting any one or any combination of organs, cavities, or body parts, which is characterized by single or multiple local abnormal proliferations of cells, groups of cells, or tissues, whether benign or malignant.

[0706] Any amount of the polynucleotides of the present invention may be administered as long as it has a biologically inhibiting effect on the proliferation of the treated cells. Moreover, it is possible to administer more than one of the polynucleotide of the present invention simultaneously to the same site. By "biologically inhibiting" is meant partial or total growth inhibition as well as decreases in the rate of proliferation or growth of the cells. The biologically inhibitory dose may be determined by assessing the effects of the polynucleotides of the present invention on target malignant or abnormally proliferating cell growth in tissue culture, tumor growth in animals and cell cultures, or any other method known to one of ordinary skill in the art.

[0707] Moreover, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention of the present invention are useful in inhibiting the angiogenesis of proliferative cells or tissues, either alone, as a protein fusion, or in combination with other polypeptides directly or indirectly, as described elsewhere herein. In a most preferred embodiment, said anti-angiogenesis effect may be achieved indirectly, for example, through the inhibition of hematopoietic, tumor-specific cells, such as tumor-associated macrophages (See Joseph IB, et al. J Natl Cancer Inst, 90(21):1648-53 (1998), which is hereby incorporated by reference).

[0708] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in inhibiting proliferative cells or tissues through the induction of apoptosis. These fusion protieins and/or polynucleotides may act either directly, or indirectly to induce apoptosis of proliferative cells and tissues, for example in the activation of a death-domain receptor, such as tumor necrosis factor (TNF) receptor-1, CD95 (Fas/APO-1), TNF-receptor-related apoptosis-mediated protein (TRAMP) and TNF-related apoptosis-inducing ligand (TRAIL) receptor-1 and -2 (See Schulze-Osthoff K, et.al., Eur J Biochem 254(3):439-59 (1998), which is hereby incorporated by reference).

Moreover, in another preferred embodiment of the present invention, these fusion proteins and/or polynucleotides may induce apoptosis through other mechanisms, such as in the activation of other proteins which will activate apoptosis, or through stimulating the expression of these proteins, either alone or in combination with small molecule drugs or adjuviants, such as apoptonin, galectins, thioredoxins, anti-inflammatory proteins (See for example, Mutat Res 400(1-2):447-55 (1998), Med Hypotheses.50(5):423-33 (1998), Chem Biol Interact. Apr 24;111-112:23-34 (1998), J Mol Med.76(6):402-12 (1998), Int J Tissue React;20(1):3-15 (1998), which are all hereby incorporated by reference).

[0709] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are useful in inhibiting the metastasis of proliferative cells or tissues. Inhibition may occur as a direct result of administering these albumin fusion proteins and/or polynucleotides, or indirectly, such as activating the expression of proteins known to inhibit metastasis, for example alpha 4 integrins, (See, e.g., Curr Top Microbiol Immunol 1998;231:125-41, which is hereby incorporated by reference). Such thereapeutic affects of the present invention may be achieved either alone, or in combination with small molecule drugs or adjuvants.

[0710] In another embodiment, the invention provides a method of delivering compositions containing the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention to targeted cells expressing the a polypeptide bound by, that binds to, or associates with an albumin fuison protein of the invention. Albumin fusion proteins of the invention may be associated with with heterologous polypeptides, heterologous nucleic acids, toxins, or prodrugs via hydrophobic, hydrophilic, ionic and/or covalent interactions.

[0711] Albumin fusion proteins of the invention are useful in enhancing the immunogenicity and/or antigenicity of proliferating cells or tissues, either directly, such as would occur if the albumin fusion proteins of the invention 'vaccinated' the immune response to respond to proliferative antigens and immunogens, or indirectly, such as in activating the expression of proteins known to enhance the immune response (e.g. chemokines), to said antigens and immunogens.

Renal Disorders

[0712] Albumin fusion proteins of the invention and/or polynucleotides encoding

albumin fusion proteins of the invention, may be used to treat, prevent, diagnose, and/or prognose disorders of the renal system. Renal disorders which can be diagnosed, prognosed, prevented, and/or treated with compositions of the invention include, but are not limited to, kidney failure, nephritis, blood vessel disorders of kidney, metabolic and congenital kidney disorders, urinary disorders of the kidney, autoimmune disorders, sclerosis and necrosis, electrolyte imbalance, and kidney cancers.

[0713] Kidney diseases which can be diagnosed, prognosed, prevented, and/or treated with compositions of the invention include, but are not limited to, acute kidney failure, chronic kidney failure, atheroembolic renal failure, end-stage renal disease, inflammatory diseases of the kidney (e.g., acute glomerulonephritis, postinfectious glomerulonephritis, rapidly progressive glomerulonephritis, nephrotic syndrome, membranous glomerulonephritis, familial nephrotic syndrome, membranoproliferative glomerulonephritis I and II, mesangial proliferative glomerulonephritis, chronic glomerulonephritis, acute tubulointerstitial nephritis, chronic tubulointerstitial nephritis, acute post-streptococcal glomerulonephritis (PSGN), pyelonephritis, lupus nephritis, chronic nephritis, interstitial nephritis, and post-streptococcal glomerulonephritis), blood vessel disorders of the kidneys (e.g., kidney infarction, atheroembolic kidney disease, cortical necrosis, malignant nephrosclerosis, renal vein thrombosis, renal underperfusion, renal retinopathy, renal ischemia-reperfusion, renal artery embolism, and renal artery stenosis), and kidney disorders resulting form urinary tract disease (e.g., pyelonephritis, hydronephrosis, urolithiasis (renal lithiasis, nephrolithiasis), reflux nephropathy, urinary tract infections, urinary retention, and acute or chronic unilateral obstructive uropathy.)

[0714] In addition, compositions of the invention can be used to diagnose, prognose, prevent, and/or treat metabolic and congenital disorders of the kidney (e.g., uremia, renal amyloidosis, renal osteodystrophy, renal tubular acidosis, renal glycosuria, nephrogenic diabetes insipidus, cystinuria, Fanconi's syndrome, renal fibrocystic osteosis (renal rickets), Hartnup disease, Bartter's syndrome, Liddle's syndrome, polycystic kidney disease, medullary cystic disease, medullary sponge kidney, Alport's syndrome, nail-patella syndrome, congenital nephrotic syndrome, CRUSH syndrome, horseshoe kidney, diabetic nephropathy, nephrogenic diabetes insipidus, analgesic nephropathy, kidney stones, and membranous nephropathy), and autoimmune disorders of the kidney (e.g., systemic lupus erythematosus (SLE), Goodpasture syndrome, IgA nephropathy, and IgM mesangial proliferative glomerulonephritis).

[0715] Compositions of the invention can also be used to diagnose, prognose, prevent, and/or treat sclerotic or necrotic disorders of the kidney (e.g., glomerulosclerosis, diabetic nephropathy, focal segmental glomerulosclerosis (FSGS), necrotizing glomerulonephritis, and renal papillary necrosis), cancers of the kidney (e.g., nephroma, hypernephroma, nephroblastoma, renal cell cancer, transitional cell cancer, renal adenocarcinoma, squamous cell cancer, and Wilm's tumor), and electrolyte imbalances (e.g., nephrocalcinosis, pyuria, edema, hydronephritis, proteinuria, hyponatremia, hypernatremia, hypokalemia, hyperkalemia, hypocalcemia, hypercalcemia, hypophosphatemia, and hyperphosphatemia).

[0716] Compositions of the invention may be administered using any method known in the art, including, but not limited to, direct needle injection at the delivery site, intravenous injection, topical administration, catheter infusion, biolistic injectors, particle accelerators, gelfoam sponge depots, other commercially available depot materials, osmotic pumps, oral or suppositorial solid pharmaceutical formulations, decanting or topical applications during surgery, aerosol delivery. Such methods are known in the art. Compositions of the invention may be administered as part of a Therapeutic, described in more detail below. Methods of delivering polynucleotides of the invention are described in more detail herein.

Cardiovascular Disorders

[0717] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, may be used to treat, prevent, diagnose, and/or prognose cardiovascular disorders, including, but not limited to, peripheral artery disease, such as limb ischemia.

[0718] Cardiovascular disorders include, but are not limited to, cardiovascular abnormalities, such as arterio-arterial fistula, arteriovenous fistula, cerebral arteriovenous malformations, congenital heart defects, pulmonary atresia, and Scimitar Syndrome. Congenital heart defects include, but are not limited to, aortic coarctation, cor triatriatum, coronary vessel anomalies, crisscross heart, dextrocardia, patent ductus arteriosus, Ebstein's anomaly, Eisenmenger complex, hypoplastic left heart syndrome, levocardia, tetralogy of fallot, transposition of great vessels, double outlet right ventricle, tricuspid atresia, persistent truncus arteriosus, and heart septal defects, such as aortopulmonary septal defect, endocardial cushion defects, Lutembacher's Syndrome, trilogy of Fallot, ventricular heart septal defects.

[0719] Cardiovascular disorders also include, but are not limited to, heart disease, such as arrhythmias, carcinoid heart disease, high cardiac output, low cardiac output, cardiac tamponade, endocarditis (including bacterial), heart aneurysm, cardiac arrest, congestive heart failure, congestive cardiomyopathy, paroxysmal dyspnea, cardiac edema, heart hypertrophy, congestive cardiomyopathy, left ventricular hypertrophy, right ventricular hypertrophy, post-infarction heart rupture, ventricular septal rupture, heart valve diseases, myocardial diseases, myocardial ischemia, pericardial effusion, pericarditis (including constrictive and tuberculous), pneumopericardium, postpericardiotomy syndrome, pulmonary heart disease, rheumatic heart disease, ventricular dysfunction, hyperemia, cardiovascular pregnancy complications, Scimitar Syndrome, cardiovascular syphilis, and cardiovascular tuberculosis.

[0720] Arrhythmias include, but are not limited to, sinus arrhythmia, atrial fibrillation, atrial flutter, bradycardia, extrasystole, Adams-Stokes Syndrome, bundle-branch block, sinoatrial block, long QT syndrome, parasystole, Lown-Ganong-Levine Syndrome, Mahaim-type pre-excitation syndrome, Wolff-Parkinson-White syndrome, sick sinus syndrome, tachycardias, and ventricular fibrillation. Tachycardias include paroxysmal tachycardia, supraventricular tachycardia, accelerated idioventricular rhythm, atrioventricular nodal reentry tachycardia, ectopic atrial tachycardia, ectopic junctional tachycardia, sinoatrial nodal reentry tachycardia, sinus tachycardia, Torsades de Pointes, and ventricular tachycardia.

[0721] Heart valve diseases include, but are not limited to, aortic valve insufficiency, aortic valve stenosis, hear murmurs, aortic valve prolapse, mitral valve prolapse, tricuspid valve prolapse, mitral valve insufficiency, mitral valve stenosis, pulmonary atresia, pulmonary valve insufficiency, pulmonary valve stenosis, tricuspid atresia, tricuspid valve insufficiency, and tricuspid valve stenosis.

[0722] Myocardial diseases include, but are not limited to, alcoholic cardiomyopathy, congestive cardiomyopathy, hypertrophic cardiomyopathy, aortic subvalvular stenosis, pulmonary subvalvular stenosis, restrictive cardiomyopathy, Chagas cardiomyopathy, endocardial fibroelastosis, endomyocardial fibrosis, Kearns Syndrome, myocardial reperfusion injury, and myocarditis.

[0723] Myocardial ischemias include, but are not limited to, coronary disease, such as angina pectoris, coronary aneurysm, coronary arteriosclerosis, coronary thrombosis, coronary vasospasm, myocardial infarction and myocardial stunning.

[0724] Cardiovascular diseases also include vascular diseases such as aneurysms, angiodysplasia, angiomatosis, bacillary angiomatosis, Hippel-Lindau Disease, Klippel-

Trenaunay-Weber Syndrome, Sturge-Weber Syndrome, angioneurotic edema, aortic diseases, Takayasu's Arteritis, aortitis, Leriche's Syndrome, arterial occlusive diseases, arteritis, enarteritis, polyarteritis nodosa, cerebrovascular disorders, diabetic angiopathies, diabetic retinopathy, embolisms, thrombosis, erythromelalgia, hemorrhoids, hepatic veno-occlusive disease, hypertension, hypotension, ischemia, peripheral vascular diseases, phlebitis, pulmonary veno-occlusive disease, Raynaud's disease, CREST syndrome, retinal vein occlusion, Scimitar syndrome, superior vena cava syndrome, telangiectasia, atacia telangiectasia, hereditary hemorrhagic telangiectasia, varicocele, varicose veins, varicose ulcer, vasculitis, and venous insufficiency.

[0725] Aneurysms include, but are not limited to, dissecting aneurysms, false aneurysms, infected aneurysms, ruptured aneurysms, aortic aneurysms, cerebral aneurysms, coronary aneurysms, heart aneurysms, and iliac aneurysms.

[0726] Arterial occlusive diseases include, but are not limited to, arteriosclerosis, intermittent claudication, carotid stenosis, fibromuscular dysplasias, mesenteric vascular occlusion, Moyamoya disease, renal artery obstruction, retinal artery occlusion, and thromboangiitis obliterans.

[0727] Cerebrovascular disorders include, but are not limited to, carotid artery diseases, cerebral amyloid angiopathy, cerebral aneurysm, cerebral anoxia, cerebral arteriosclerosis, cerebral arteriovenous malformation, cerebral artery diseases, cerebral embolism and thrombosis, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, cerebral hemorrhage, epidural hematoma, subdural hematoma, subaraxhnoid hemorrhage, cerebral infarction, cerebral ischemia (including transient), subclavian steal syndrome, periventricular leukomalacia, vascular headache, cluster headache, migraine, and vertebrobasilar insufficiency.

[0728] Embolisms include, but are not limited to, air embolisms, amniotic fluid embolisms, cholesterol embolisms, blue toe syndrome, fat embolisms, pulmonary embolisms, and thromoboembolisms. Thrombosis include, but are not limited to, coronary thrombosis, hepatic vein thrombosis, retinal vein occlusion, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, and thrombophlebitis.

[0729] Ischemic disorders include, but are not limited to, cerebral ischemia, ischemic colitis, compartment syndromes, anterior compartment syndrome, myocardial ischemia, reperfusion injuries, and peripheral limb ischemia. Vasculitis includes, but is not limited to, aortitis, arteritis, Behcet's Syndrome, Churg-Strauss Syndrome, mucocutaneous lymph node

syndrome, thromboangiitis obliterans, hypersensitivity vasculitis, Schoenlein-Henoch purpura, allergic cutaneous vasculitis, and Wegener's granulomatosis.

[0730] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be administered using any method known in the art, including, but not limited to, direct needle injection at the delivery site, intravenous injection, topical administration, catheter infusion, biolistic injectors, particle accelerators, gelfoam sponge depots, other commercially available depot materials, osmotic pumps, oral or suppositorial solid pharmaceutical formulations, decanting or topical applications during surgery, aerosol delivery. Such methods are known in the art. Methods of delivering polynucleotides are described in more detail herein.

Respiratory Disorders

[0731] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used to treat, prevent, diagnose, and/or prognose diseases and/or disorders of the respiratory system.

[0732] Diseases and disorders of the respiratory system include, but are not limited to, nasal vestibulitis, nonallergic rhinitis (e.g., acute rhinitis, chronic rhinitis, atrophic rhinitis, vasomotor rhinitis), nasal polyps, and sinusitis, juvenile angiofibromas, cancer of the nose and juvenile papillomas, vocal cord polyps, nodules (singer's nodules), contact ulcers, vocal cord paralysis, laryngoceles, pharyngitis (e.g., viral and bacterial), tonsillitis, tonsillar cellulitis, parapharyngeal abscess, laryngitis, laryngoceles, and throat cancers (e.g., cancer of the nasopharynx, tonsil cancer, larynx cancer), lung cancer (e.g., squamous cell carcinoma, small cell (oat cell) carcinoma, large cell carcinoma, and adenocarcinoma), allergic disorders (eosinophilic pneumonia, hypersensitivity pneumonitis (e.g., extrinsic allergic alveolitis, allergic interstitial pneumonitis, organic dust pneumoconiosis, allergic bronchopulmonary aspergillosis, asthma, Wegener's granulomatosis (granulomatous vasculitis), Goodpasture's syndrome)), pneumonia (e.g., bacterial pneumonia (e.g., *Streptococcus pneumoniae* (pneumoncoccal pneumonia), *Staphylococcus aureus* (staphylococcal pneumonia), Gram-negative bacterial pneumonia (caused by, e.g., *Klebsiella* and *Pseudomas spp.*), *Mycoplasma pneumoniae* pneumonia, *Hemophilus influenzae* pneumonia, *Legionella pneumophila* (Legionnaires' disease), and *Chlamydia psittaci* (Psittacosis)), and viral pneumonia (e.g., influenza, chickenpox (varicella).

[0733] Additional diseases and disorders of the respiratory system include, but are not limited to bronchiolitis, polio (poliomyelitis), croup, respiratory syncytial viral infection, mumps, erythema infectiosum (fifth disease), roseola infantum, progressive rubella panencephalitis, german measles, and subacute sclerosing panencephalitis), fungal pneumonia (e.g., Histoplasmosis, Coccidioidomycosis, Blastomycosis, fungal infections in people with severely suppressed immune systems (e.g., cryptococcosis, caused by *Cryptococcus neoformans*; aspergillosis, caused by *Aspergillus spp.*; candidiasis, caused by *Candida*; and mucormycosis)), *Pneumocystis carinii* (pneumocystis pneumonia), atypical pneumonias (e.g., *Mycoplasma* and *Chlamydia* spp.), opportunistic infection pneumonia, nosocomial pneumonia, chemical pneumonitis, and aspiration pneumonia, pleural disorders (e.g., pleurisy, pleural effusion, and pneumothorax (e.g., simple spontaneous pneumothorax, complicated spontaneous pneumothorax, tension pneumothorax)), obstructive airway diseases (e.g., asthma, chronic obstructive pulmonary disease (COPD), emphysema, chronic or acute bronchitis), occupational lung diseases (e.g., silicosis, black lung (coal workers' pneumoconiosis), asbestosis, berylliosis, occupational asthsma, byssinosis, and benign pneumoconioses), Infiltrative Lung Disease (e.g., pulmonary fibrosis (e.g., fibrosing alveolitis, usual interstitial pneumonia), idiopathic pulmonary fibrosis, desquamative interstitial pneumonia, lymphoid interstitial pneumonia, histiocytosis X (e.g., Letterer-Siwe disease, Hand-Schüller-Christian disease, eosinophilic granuloma), idiopathic pulmonary hemosiderosis, sarcoidosis and pulmonary alveolar proteinosis), Acute respiratory distress syndrome (also called, e.g., adult respiratory distress syndrome), edema, pulmonary embolism, bronchitis (e.g., viral, bacterial), bronchiectasis, atelectasis, lung abscess (caused by, e.g., *Staphylococcus aureus* or *Legionella pneumophila*), and cystic fibrosis.

Anti-Angiogenesis Activity

[0734] The naturally occurring balance between endogenous stimulators and inhibitors of angiogenesis is one in which inhibitory influences predominate. Rastinejad *et al., Cell 56*:345-355 (1989). In those rare instances in which neovascularization occurs under normal physiological conditions, such as wound healing, organ regeneration, embryonic development, and female reproductive processes, angiogenesis is stringently regulated and spatially and temporally delimited. Under conditions of pathological angiogenesis such as that characterizing solid tumor growth, these regulatory controls fail. Unregulated

angiogenesis becomes pathologic and sustains progression of many neoplastic and non-neoplastic diseases. A number of serious diseases are dominated by abnormal neovascularization including solid tumor growth and metastases, arthritis, some types of eye disorders, and psoriasis. See, e.g., reviews by Moses *et al., Biotech. 9*:630-634 (1991); Folkman *et al., N. Engl. J. Med., 333*:1757-1763 (1995); Auerbach *et al., J. Microvasc. Res. 29*:401-411 (1985); Folkman, Advances in Cancer Research, eds. Klein and Weinhouse, Academic Press, New York, pp. 175-203 (1985); Patz, *Am. J. Opthalmol. 94*:715-743 (1982); and Folkman *et al.,* Science *221*:719-725 (1983). In a number of pathological conditions, the process of angiogenesis contributes to the disease state. For example, significant data have accumulated which suggest that the growth of solid tumors is dependent on angiogenesis. Folkman and Klagsbrun, *Science 235*:442-447 (1987).

[0735] The present invention provides for treatment of diseases or disorders associated with neovascularization by administration of fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention. Malignant and metastatic conditions which can be treated with the polynucleotides and polypeptides, or agonists or antagonists of the invention include, but are not limited to, malignancies, solid tumors, and cancers described herein and otherwise known in the art (for a review of such disorders, see Fishman *et al.,* Medicine, 2d Ed., J. B. Lippincott Co., Philadelphia (1985)).Thus, the present invention provides a method of treating an angiogenesis-related disease and/or disorder, comprising administering to an individual in need thereof a therapeutically effective amount of an albumin fusion protein of the invention and/or polynucleotides encoding an albumin fusion protein of the invention. For example, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be utilized in a variety of additional methods in order to therapeutically treat a cancer or tumor. Cancers which may be treated with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to solid tumors, including prostate, lung, breast, ovarian, stomach, pancreas, larynx, esophagus, testes, liver, parotid, biliary tract, colon, rectum, cervix, uterus, endometrium, kidney, bladder, thyroid cancer; primary tumors and metastases; melanomas; glioblastoma; Kaposi's sarcoma; leiomyosarcoma; non- small cell lung cancer; colorectal cancer; advanced malignancies; and blood born tumors such as leukemias. For example, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be delivered topically, in order to treat cancers such as skin cancer, head and neck tumors, breast

tumors, and Kaposi's sarcoma.

[0736] Within yet other aspects, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be utilized to treat superficial forms of bladder cancer by, for example, intravesical administration. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be delivered directly into the tumor, or near the tumor site, via injection or a catheter. Of course, as the artisan of ordinary skill will appreciate, the appropriate mode of administration will vary according to the cancer to be treated. Other modes of delivery are discussed herein.

[0737] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be useful in treating other disorders, besides cancers, which involve angiogenesis. These disorders include, but are not limited to: benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; artheroscleric plaques; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis, retinoblastoma, uvietis and Pterygia (abnormal blood vessel growth) of the eye; rheumatoid arthritis; psoriasis; delayed wound healing; endometriosis; vasculogenesis; granulations; hypertrophic scars (keloids); nonunion fractures; scleroderma; trachoma; vascular adhesions; myocardial angiogenesis; coronary collaterals; cerebral collaterals; arteriovenous malformations; ischemic limb angiogenesis; Osler-Webber Syndrome; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; fibromuscular dysplasia; wound granulation; Crohn's disease; and atherosclerosis.

[0738] For example, within one aspect of the present invention methods are provided for treating hypertrophic scars and keloids, comprising the step of administering albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention to a hypertrophic scar or keloid.

[0739] Within one embodiment of the present invention fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are directly injected into a hypertrophic scar or keloid, in order to prevent the progression of these lesions. This therapy is of particular value in the prophylactic treatment of conditions which are known to result in the development of hypertrophic scars and keloids (e.g., burns), and is preferably initiated after the proliferative phase has had time to progress

(approximately 14 days after the initial injury), but before hypertrophic scar or keloid development. As noted above, the present invention also provides methods for treating neovascular diseases of the eye, including for example, corneal neovascularization, neovascular glaucoma, proliferative diabetic retinopathy, retrolental fibroplasia and macular degeneration.

[0740] Moreover, Ocular disorders associated with neovascularization which can be treated with the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to: neovascular glaucoma, diabetic retinopathy, retinoblastoma, retrolental fibroplasia, uveitis, retinopathy of prematurity macular degeneration, corneal graft neovascularization, as well as other eye inflammatory diseases, ocular tumors and diseases associated with choroidal or iris neovascularization. See, e.g., reviews by Waltman *et al., Am. J. Ophthal. 85*:704-710 (1978) and Gartner *et al., Surv. Ophthal. 22*:291-312 (1978).

[0741] Thus, within one aspect of the present invention methods are provided for treating neovascular diseases of the eye such as corneal neovascularization (including corneal graft neovascularization), comprising the step of administering to a patient a therapeutically effective amount of a compound (e.g., fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention) to the cornea, such that the formation of blood vessels is inhibited. Briefly, the cornea is a tissue which normally lacks blood vessels. In certain pathological conditions however, capillaries may extend into the cornea from the pericorneal vascular plexus of the limbus. When the cornea becomes vascularized, it also becomes clouded, resulting in a decline in the patient's visual acuity. Visual loss may become complete if the cornea completely opacitates. A wide variety of disorders can result in corneal neovascularization, including for example, corneal infections (e.g., trachoma, herpes simplex keratitis, leishmaniasis and onchocerciasis), immunological processes (e.g., graft rejection and Stevens-Johnson's syndrome), alkali burns, trauma, inflammation (of any cause), toxic and nutritional deficiency states, and as a complication of wearing contact lenses.

[0742] Within particularly preferred embodiments of the invention, may be prepared for topical administration in saline (combined with any of the preservatives and antimicrobial agents commonly used in ocular preparations), and administered in eyedrop form. The solution or suspension may be prepared in its pure form and administered several times daily. Alternatively, anti-angiogenic compositions, prepared as described above, may also be

administered directly to the cornea. Within preferred embodiments, the anti-angiogenic composition is prepared with a muco-adhesive polymer which binds to cornea. Within further embodiments, the anti-angiogenic factors or anti-angiogenic compositions may be utilized as an adjunct to conventional steroid therapy. Topical therapy may also be useful prophylactically in corneal lesions which are known to have a high probability of inducing an angiogenic response (such as chemical burns). In these instances the treatment, likely in combination with steroids, may be instituted immediately to help prevent subsequent complications.

[0743] Within other embodiments, the compounds described above may be injected directly into the corneal stroma by an ophthalmologist under microscopic guidance. The preferred site of injection may vary with the morphology of the individual lesion, but the goal of the administration would be to place the composition at the advancing front of the vasculature (i.e., interspersed between the blood vessels and the normal cornea). In most cases this would involve perilimbic corneal injection to "protect" the cornea from the advancing blood vessels. This method may also be utilized shortly after a corneal insult in order to prophylactically prevent corneal neovascularization. In this situation the material could be injected in the perilimbic cornea interspersed between the corneal lesion and its undesired potential limbic blood supply. Such methods may also be utilized in a similar fashion to prevent capillary invasion of transplanted corneas. In a sustained-release form injections might only be required 2-3 times per year. A steroid could also be added to the injection solution to reduce inflammation resulting from the injection itself.

[0744] Within another aspect of the present invention, methods are provided for treating neovascular glaucoma, comprising the step of administering to a patient a therapeutically effective amount of an albumin fusion protein of the invention and/or polynucleotides encoding an albumin fusion protein of the invention to the eye, such that the formation of blood vessels is inhibited. In one embodiment, the compound may be administered topically to the eye in order to treat early forms of neovascular glaucoma. Within other embodiments, the compound may be implanted by injection into the region of the anterior chamber angle. Within other embodiments, the compound may also be placed in any location such that the compound is continuously released into the aqueous humor. Within another aspect of the present invention, methods are provided for treating proliferative diabetic retinopathy, comprising the step of administering to a patient a therapeutically effective amount of an albumin fusion protein of the invention and/or

polynucleotides encoding an albumin fusion protein of the invention to the eyes, such that the formation of blood vessels is inhibited.

[0745] Within particularly preferred embodiments of the invention, proliferative diabetic retinopathy may be treated by injection into the aqueous humor or the vitreous, in order to increase the local concentration of the polynucleotide, polypeptide, antagonist and/or agonist in the retina. Preferably, this treatment should be initiated prior to the acquisition of severe disease requiring photocoagulation.

[0746] Within another aspect of the present invention, methods are provided for treating retrolental fibroplasia, comprising the step of administering to a patient a therapeutically effective amount of an albumin fusion protein of the invention and/or polynucleotides encoding an albumin fusion protein of the invention to the eye, such that the formation of blood vessels is inhibited. The compound may be administered topically, via intravitreous injection and/or via intraocular implants.

[0747] Additionally, disorders which can be treated with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to, hemangioma, arthritis, psoriasis, angiofibroma, atherosclerotic plaques, delayed wound healing, granulations, hemophilic joints, hypertrophic scars, nonunion fractures, Osler-Weber syndrome, pyogenic granuloma, scleroderma, trachoma, and vascular adhesions.

[0748] Moreover, disorders and/or states, which can be treated, prevented, diagnosed, and/or prognosed with the the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention of the invention include, but are not limited to, solid tumors, blood born tumors such as leukemias, tumor metastasis, Kaposi's sarcoma, benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas, rheumatoid arthritis, psoriasis, ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis, retinoblastoma, and uvietis, delayed wound healing, endometriosis, vascluogenesis, granulations, hypertrophic scars (keloids), nonunion fractures, scleroderma, trachoma, vascular adhesions, myocardial angiogenesis, coronary collaterals, cerebral collaterals, arteriovenous malformations, ischemic limb angiogenesis, Osler-Webber Syndrome, plaque neovascularization, telangiectasia, hemophiliac joints, angiofibroma fibromuscular dysplasia, wound granulation, Crohn's disease, atherosclerosis, birth control agent by preventing

vascularization required for embryo implantation controlling menstruation, diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (Rochele minalia quintosa), ulcers (Helicobacter pylori), Bartonellosis and bacillary angiomatosis.

[0749] In one aspect of the birth control method, an amount of the compound sufficient to block embryo implantation is administered before or after intercourse and fertilization have occurred, thus providing an effective method of birth control, possibly a "morning after" method. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may also be used in controlling menstruation or administered as either a peritoneal lavage fluid or for peritoneal implantation in the treatment of endometriosis.

[0750] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be incorporated into surgical sutures in order to prevent stitch granulomas.

[0751] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be utilized in a wide variety of surgical procedures. For example, within one aspect of the present invention a compositions (in the form of, for example, a spray or film) may be utilized to coat or spray an area prior to removal of a tumor, in order to isolate normal surrounding tissues from malignant tissue, and/or to prevent the spread of disease to surrounding tissues. Within other aspects of the present invention, compositions (e.g., in the form of a spray) may be delivered via endoscopic procedures in order to coat tumors, or inhibit angiogenesis in a desired locale. Within yet other aspects of the present invention, surgical meshes which have been coated with anti-angiogenic compositions of the present invention may be utilized in any procedure wherein a surgical mesh might be utilized. For example, within one embodiment of the invention a surgical mesh laden with an anti-angiogenic composition may be utilized during abdominal cancer resection surgery (e.g., subsequent to colon resection) in order to provide support to the structure, and to release an amount of the anti-angiogenic factor.

[0752] Within further aspects of the present invention, methods are provided for treating tumor excision sites, comprising administering albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention to the resection margins of a tumor subsequent to excision, such that the local recurrence of cancer and the formation of new blood vessels at the site is inhibited. Within one embodiment of the invention, the anti-angiogenic compound is administered directly to the tumor excision site

(e.g., applied by swabbing, brushing or otherwise coating the resection margins of the tumor with the anti-angiogenic compound). Alternatively, the anti-angiogenic compounds may be incorporated into known surgical pastes prior to administration. Within particularly preferred embodiments of the invention, the anti-angiogenic compounds are applied after hepatic resections for malignancy, and after neurosurgical operations.

[0753] Within one aspect of the present invention, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be administered to the resection margin of a wide variety of tumors, including for example, breast, colon, brain and hepatic tumors. For example, within one embodiment of the invention, anti-angiogenic compounds may be administered to the site of a neurological tumor subsequent to excision, such that the formation of new blood vessels at the site are inhibited.

[0754] The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may also be administered along with other anti-angiogenic factors. Representative examples of other anti-angiogenic factors include: Anti-Invasive Factor, retinoic acid and derivatives thereof, paclitaxel, Suramin, Tissue Inhibitor of Metalloproteinase-1, Tissue Inhibitor of Metalloproteinase-2, Plasminogen Activator Inhibitor-1, Plasminogen Activator Inhibitor-2, and various forms of the lighter "d group" transition metals.

[0755] Lighter "d group" transition metals include, for example, vanadium, molybdenum, tungsten, titanium, niobium, and tantalum species. Such transition metal species may form transition metal complexes. Suitable complexes of the above-mentioned transition metal species include oxo transition metal complexes.

[0756] Representative examples of vanadium complexes include oxo vanadium complexes such as vanadate and vanadyl complexes. Suitable vanadate complexes include metavanadate and orthovanadate complexes such as, for example, ammonium metavanadate, sodium metavanadate, and sodium orthovanadate. Suitable vanadyl complexes include, for example, vanadyl acetylacetonate and vanadyl sulfate including vanadyl sulfate hydrates such as vanadyl sulfate mono- and trihydrates.

[0757] Representative examples of tungsten and molybdenum complexes also include oxo complexes. Suitable oxo tungsten complexes include tungstate and tungsten oxide complexes. Suitable tungstate complexes include ammonium tungstate, calcium tungstate, sodium tungstate dihydrate, and tungstic acid. Suitable tungsten oxides include tungsten (IV)

oxide and tungsten (VI) oxide. Suitable oxo molybdenum complexes include molybdate, molybdenum oxide, and molybdenyl complexes. Suitable molybdate complexes include ammonium molybdate and its hydrates, sodium molybdate and its hydrates, and potassium molybdate and its hydrates. Suitable molybdenum oxides include molybdenum (VI) oxide, molybdenum (VI) oxide, and molybdic acid. Suitable molybdenyl complexes include, for example, molybdenyl acetylacetonate. Other suitable tungsten and molybdenum complexes include hydroxo derivatives derived from, for example, glycerol, tartaric acid, and sugars.

[0758] A wide variety of other anti-angiogenic factors may also be utilized within the context of the present invention. Representative examples include platelet factor 4; protamine sulphate; sulphated chitin derivatives (prepared from queen crab shells), (Murata et al., Cancer Res. 51:22-26, 1991); Sulphated Polysaccharide Peptidoglycan Complex (SP- PG) (the function of this compound may be enhanced by the presence of steroids such as estrogen, and tamoxifen citrate); Staurosporine; modulators of matrix metabolism, including for example, proline analogs, cishydroxyproline, d,L-3,4-dehydroproline, Thiaproline, alpha,alpha-dipyridyl, aminopropionitrile fumarate; 4-propyl-5-(4-pyridinyl)-2(3H)-oxazolone; Methotrexate; Mitoxantrone; Heparin; Interferons; 2 Macroglobulin-serum; ChIMP-3 (Pavloff et al., J. Bio. Chem. 267:17321-17326, (1992)); Chymostatin (Tomkinson et al., Biochem J. 286:475-480, (1992)); Cyclodextrin Tetradecasulfate; Eponemycin; Camptothecin; Fumagillin (Ingber et al., Nature 348:555-557, 1990); Gold Sodium Thiomalate ("GST"; Matsubara and Ziff, J. Clin. Invest. 79:1440-1446, (1987)); anticollagenase-serum; alpha2-antiplasmin (Holmes et al., J. Biol. Chem. 262(4):1659-1664, (1987)); Bisantrene (National Cancer Institute); Lobenzarit disodium (N-(2)-carboxyphenyl-4- chloroanthronilic acid disodium or "CCA"; Takeuchi et al., Agents Actions 36:312-316, (1992)); Thalidomide; Angostatic steroid; AGM-1470; carboxynaminolmidazole; and metalloproteinase inhibitors such as BB94.

Diseases at the Cellular Level

[0759] Diseases associated with increased cell survival or the inhibition of apoptosis that could be treated, prevented, diagnosed, and/or prognosed using fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, including, but not limited to colon cancer, cardiac tumors, pancreatic cancer, melanoma, retinoblastoma, glioblastoma, lung cancer, intestinal cancer, testicular

cancer, stomach cancer, neuroblastoma, myxoma, myoma, lymphoma, endothelioma, osteoblastoma, osteoclastoma, osteosarcoma, chondrosarcoma, adenoma, breast cancer, prostate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis and rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), inflammation, graft v. host disease, acute graft rejection, and chronic graft rejection.

[0760] In preferred embodiments, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to inhibit growth, progression, and/or metasis of cancers, in particular those listed above.

[0761] Additional diseases or conditions associated with increased cell survival that could be treated or detected by fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as leukemia (including acute leukemias (e.g., acute lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (e.g., chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia)), polycythemia vera, lymphomas (e.g., Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma, and retinoblastoma.

[0762] Diseases associated with increased apoptosis that could be treated, prevented, diagnosed, and/or prognesed using fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, include, but are not limited to, AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration and brain tumor or prior associated disease); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis and rheumatoid arthritis) myelodysplastic syndromes (such as aplastic anemia), graft v. host disease, ischemic injury (such as that caused by myocardial infarction, stroke and reperfusion injury), liver injury (e.g., hepatitis related liver injury, ischemia/reperfusion injury, cholestosis (bile duct injury) and liver cancer); toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia and anorexia.

Wound Healing and Epithelial Cell Proliferation

[0763] In accordance with yet a further aspect of the present invention, there is provided a process for utilizing fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, for therapeutic purposes, for example, to stimulate epithelial cell proliferation and basal keratinocytes for the purpose of wound healing, and to stimulate hair follicle production and healing of dermal wounds. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, may be clinically useful in stimulating wound healing including surgical wounds, excisional wounds, deep wounds involving damage of the dermis and epidermis, eye tissue wounds, dental tissue wounds, oral cavity wounds, diabetic ulcers, dermal ulcers, cubitus ulcers, arterial ulcers, venous stasis ulcers, burns resulting from heat exposure or chemicals, and other abnormal wound healing conditions such as uremia, malnutrition, vitamin deficiencies and complications associated with systemic treatment with steroids, radiation therapy and antineoplastic drugs and antimetabolites. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could be used to promote dermal reestablishment subsequent to dermal loss

[0764] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could be used to increase the adherence of skin grafts to a wound bed and to stimulate re-epithelialization from the wound bed. The

following are types of grafts that fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could be used to increase adherence to a wound bed: autografts, artificial skin, allografts, autodermic graft, autoepdermic grafts, avacular grafts, Blair-Brown grafts, bone graft, brephoplastic grafts, cutis graft, delayed graft, dermic graft, epidermic graft, fascia graft, full thickness graft, heterologous graft, xenograft, homologous graft, hyperplastic graft, lamellar graft, mesh graft, mucosal graft, Ollier-Thiersch graft, omenpal graft, patch graft, pedicle graft, penetrating graft, split skin graft, thick split graft. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, can be used to promote skin strength and to improve the appearance of aged skin.

[0765] It is believed that fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, will also produce changes in hepatocyte proliferation, and epithelial cell proliferation in the lung, breast, pancreas, stomach, small intestine, and large intestine. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could promote proliferation of epithelial cells such as sebocytes, hair follicles, hepatocytes, type II pneumocytes, mucin-producing goblet cells, and other epithelial cells and their progenitors contained within the skin, lung, liver, and gastrointestinal tract. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, may promote proliferation of endothelial cells, keratinocytes, and basal keratinocytes.

[0766] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could also be used to reduce the side effects of gut toxicity that result from radiation, chemotherapy treatments or viral infections. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, may have a cytoprotective effect on the small intestine mucosa. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, may also stimulate healing of mucositis (mouth ulcers) that result from chemotherapy and viral infections.

[0767] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could further be used in full regeneration of skin in full and partial thickness skin defects, including burns, (i.e., repopulation of hair follicles, sweat glands, and sebaceous glands), treatment of other skin defects such as psoriasis. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion

proteins of the invention, could be used to treat epidermolysis bullosa, a defect in adherence of the epidermis to the underlying dermis which results in frequent, open and painful blisters by accelerating reepithelialization of these lesions. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could also be used to treat gastric and doudenal ulcers and help heal by scar formation of the mucosal lining and regeneration of glandular mucosa and duodenal mucosal lining more rapidly. Inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, are diseases which result in destruction of the mucosal surface of the small or large intestine, respectively. Thus, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could be used to promote the resurfacing of the mucosal surface to aid more rapid healing and to prevent progression of inflammatory bowel disease. Treatment with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, is expected to have a significant effect on the production of mucus throughout the gastrointestinal tract and could be used to protect the intestinal mucosa from injurious substances that are ingested or following surgery. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could be used to treat diseases associate with the under expression.

[0768] Moreover, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could be used to prevent and heal damage to the lungs due to various pathological states. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, which could stimulate proliferation and differentiation and promote the repair of alveoli and brochiolar epithelium to prevent or treat acute or chronic lung damage. For example, emphysema, which results in the progressive loss of aveoli, and inhalation injuries, i.e., resulting from smoke inhalation and burns, that cause necrosis of the bronchiolar epithelium and alveoli could be effectively treated using polynucleotides or polypeptides, agonists or antagonists of the present invention. Also fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could be used to stimulate the proliferation of and differentiation of type II pneumocytes, which may help treat or prevent disease such as hyaline membrane diseases, such as infant respiratory distress syndrome and bronchopulmonary displasia, in premature infants.

[0769] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could stimulate the proliferation and differentiation

of hepatocytes and, thus, could be used to alleviate or treat liver diseases and pathologies such as fulminant liver failure caused by cirrhosis, liver damage caused by viral hepatitis and toxic substances (i.e., acetaminophen, carbon tetraholoride and other hepatotoxins known in the art).

[0770] In addition, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could be used treat or prevent the onset of diabetes mellitus. In patients with newly diagnosed Types I and II diabetes, where some islet cell function remains, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could be used to maintain the islet function so as to alleviate, delay or prevent permanent manifestation of the disease. Also, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, could be used as an auxiliary in islet cell transplantation to improve or promote islet cell function.

Neural Activity and Neurological Diseases

[0771] The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used for the diagnosis and/or treatment of diseases, disorders, damage or injury of the brain and/or nervous system. Nervous system disorders that can be treated with the compositions of the invention (e.g., fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention), include, but are not limited to, nervous system injuries, and diseases or disorders which result in either a disconnection of axons, a diminution or degeneration of neurons, or demyelination. Nervous system lesions which may be treated in a patient (including human and non-human mammalian patients) according to the methods of the invention, include but are not limited to, the following lesions of either the central (including spinal cord, brain) or peripheral nervous systems: (1) ischemic lesions, in which a lack of oxygen in a portion of the nervous system results in neuronal injury or death, including cerebral infarction or ischemia, or spinal cord infarction or ischemia; (2) traumatic lesions, including lesions caused by physical injury or associated with surgery, for example, lesions which sever a portion of the nervous system, or compression injuries; (3) malignant lesions, in which a portion of the nervous system is destroyed or injured by malignant tissue which is either a nervous system associated malignancy or a malignancy derived from non-nervous system tissue; (4) infectious lesions, in which a portion of the nervous system is destroyed or injured as a result of infection, for example, by an abscess or associated with infection by human immunodeficiency virus,

herpes zoster, or herpes simplex virus or with Lyme disease, tuberculosis, or syphilis; (5) degenerative lesions, in which a portion of the nervous system is destroyed or injured as a result of a degenerative process including but not limited to, degeneration associated with Parkinson's disease, Alzheimer's disease, Huntington's chorea, or amyotrophic lateral sclerosis (ALS); (6) lesions associated with nutritional diseases or disorders, in which a portion of the nervous system is destroyed or injured by a nutritional disorder or disorder of metabolism including, but not limited to, vitamin B12 deficiency, folic acid deficiency, Wernicke disease, tobacco-alcohol amblyopia, Marchiafava-Bignami disease (primary degeneration of the corpus callosum), and alcoholic cerebellar degeneration; (7) neurological lesions associated with systemic diseases including, but not limited to, diabetes (diabetic neuropathy, Bell's palsy), systemic lupus erythematosus, carcinoma, or sarcoidosis; (8) lesions caused by toxic substances including alcohol, lead, or particular neurotoxins; and (9) demyelinated lesions in which a portion of the nervous system is destroyed or injured by a demyelinating disease including, but not limited to, multiple sclerosis, human immunodeficiency virus-associated myelopathy, transverse myelopathy or various etiologies, progressive multifocal leukoencephalopathy, and central pontine myelinolysis.

[0772] In one embodiment, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to protect neural cells from the damaging effects of hypoxia. In a further preferred embodiment, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to protect neural cells from the damaging effects of cerebral hypoxia. According to this embodiment, the compositions of the invention are used to treat or prevent neural cell injury associated with cerebral hypoxia. In one non-exclusive aspect of this embodiment, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, are used to treat or prevent neural cell injury associated with cerebral ischemia. In another non-exclusive aspect of this embodiment, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to treat or prevent neural cell injury associated with cerebral infarction.

[0773] In another preferred embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to treat or prevent neural cell injury associated with a stroke. In a specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the

invention are used to treat or prevent cerebral neural cell injury associated with a stroke.

[0774] In another preferred embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to treat or prevent neural cell injury associated with a heart attack. In a specific embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to treat or prevent cerebral neural cell injury associated with a heart attack.

[0775] The compositions of the invention which are useful for treating or preventing a nervous system disorder may be selected by testing for biological activity in promoting the survival or differentiation of neurons. For example, and not by way of limitation, compositions of the invention which elicit any of the following effects may be useful according to the invention: (1) increased survival time of neurons in culture either in the presence or absence of hypoxia or hypoxic conditions; (2) increased sprouting of neurons in culture or *in vivo*; (3) increased production of a neuron-associated molecule in culture or *in vivo*, *e.g.*, choline acetyltransferase or acetylcholinesterase with respect to motor neurons; or (4) decreased symptoms of neuron dysfunction *in vivo*. Such effects may be measured by any method known in the art. In preferred, non-limiting embodiments, increased survival of neurons may routinely be measured using a method set forth herein or otherwise known in the art, such as, for example, in Zhang *et al.*, *Proc Natl Acad Sci USA* 97:3637-42 (2000) or in Arakawa *et al., J. Neurosci., 10*:3507-15 (1990); increased sprouting of neurons may be detected by methods known in the art, such as, for example, the methods set forth in Pestronk *et al., Exp. Neurol., 70*:65-82 (1980), or Brown *et al., Ann. Rev. Neurosci., 4*:17-42 (1981); increased production of neuron-associated molecules may be measured by bioassay, enzymatic assay, antibody binding, Northern blot assay, etc., using techniques known in the art and depending on the molecule to be measured; and motor neuron dysfunction may be measured by assessing the physical manifestation of motor neuron disorder, e.g., weakness, motor neuron conduction velocity, or functional disability.

[0776] In specific embodiments, motor neuron disorders that may be treated according to the invention include, but are not limited to, disorders such as infarction, infection, exposure to toxin, trauma, surgical damage, degenerative disease or malignancy that may affect motor neurons as well as other components of the nervous system, as well as disorders that selectively affect neurons such as amyotrophic lateral sclerosis, and including, but not limited to, progressive spinal muscular atrophy, progressive bulbar palsy, primary

lateral sclerosis, infantile and juvenile muscular atrophy, progressive bulbar paralysis of childhood (Fazio-Londe syndrome), poliomyelitis and the post polio syndrome, and Hereditary Motorsensory Neuropathy (Charcot-Marie-Tooth Disease).

[0777] Further, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may play a role in neuronal survival; synapse formation; conductance; neural differentiation, etc. Thus, compositions of the invention (including fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention) may be used to diagnose and/or treat or prevent diseases or disorders associated with these roles, including, but not limited to, learning and/or cognition disorders. The compositions of the invention may also be useful in the treatment or prevention of neurodegenerative disease states and/or behavioural disorders. Such neurodegenerative disease states and/or behavioral disorders include, but are not limited to, Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, Tourette Syndrome, schizophrenia, mania, dementia, paranoia, obsessive compulsive disorder, panic disorder, learning disabilities, ALS, psychoses, autism, and altered behaviors, including disorders in feeding, sleep patterns, balance, and perception. In addition, compositions of the invention may also play a role in the treatment, prevention and/or detection of developmental disorders associated with the developing embryo, or sexually-linked disorders.

[0778] Additionally, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, may be useful in protecting neural cells from diseases, damage, disorders, or injury, associated with cerebrovascular disorders including, but not limited to, carotid artery diseases (e.g., carotid artery thrombosis, carotid stenosis, or Moyamoya Disease), cerebral amyloid angiopathy, cerebral aneurysm, cerebral anoxia, cerebral arteriosclerosis, cerebral arteriovenous malformations, cerebral artery diseases, cerebral embolism and thrombosis (e.g., carotid artery thrombosis, sinus thrombosis, or Wallenberg's Syndrome), cerebral hemorrhage (e.g., epidural or subdural hematoma, or subarachnoid hemorrhage), cerebral infarction, cerebral ischemia (e.g., transient cerebral ischemia, Subclavian Steal Syndrome, or vertebrobasilar insufficiency), vascular dementia (e.g., multi-infarct), leukomalacia, periventricular, and vascular headache (e.g., cluster headache or migraines).

[0779] In accordance with yet a further aspect of the present invention, there is provided a process for utilizing fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, for therapeutic purposes, for example, to

stimulate neurological cell proliferation and/or differentiation. Therefore, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used to treat and/or detect neurologic diseases. Moreover, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, can be used as a marker or detector of a particular nervous system disease or disorder.

[0780] Examples of neurologic diseases which can be treated or detected with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, brain diseases, such as metabolic brain diseases which includes phenylketonuria such as maternal phenylketonuria, pyruvate carboxylase deficiency, pyruvate dehydrogenase complex deficiency, Wernicke's Encephalopathy, brain edema, brain neoplasms such as cerebellar neoplasms which include infratentorial neoplasms, cerebral ventricle neoplasms such as choroid plexus neoplasms, hypothalamic neoplasms, supratentorial neoplasms, canavan disease, cerebellar diseases such as cerebellar ataxia which include spinocerebellar degeneration such as ataxia telangiectasia, cerebellar dyssynergia, Friederich's Ataxia, Machado-Joseph Disease, olivopontocerebellar atrophy, cerebellar neoplasms such as infratentorial neoplasms, diffuse cerebral sclerosis such as encephalitis periaxialis, globoid cell leukodystrophy, metachromatic leukodystrophy and subacute sclerosing panencephalitis.

[0781] Additional neurologic diseases which can be treated or detected with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include cerebrovascular disorders (such as carotid artery diseases which include carotid artery thrombosis, carotid stenosis and Moyamoya Disease), cerebral amyloid angiopathy, cerebral aneurysm, cerebral anoxia, cerebral arteriosclerosis, cerebral arteriovenous malformations, cerebral artery diseases, cerebral embolism and thrombosis such as carotid artery thrombosis, sinus thrombosis and Wallenberg's Syndrome, cerebral hemorrhage such as epidural hematoma, subdural hematoma and subarachnoid hemorrhage, cerebral infarction, cerebral ischemia such as transient cerebral ischemia, Subclavian Steal Syndrome and vertebrobasilar insufficiency, vascular dementia such as multi-infarct dementia, periventricular leukomalacia, vascular headache such as cluster headache and migraine.

[0782] Additional neurologic diseases which can be treated or detected with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include dementia such as AIDS Dementia Complex, presenile dementia such as

Alzheimer's Disease and Creutzfeldt-Jakob Syndrome, senile dementia such as Alzheimer's Disease and progressive supranuclear palsy, vascular dementia such as multi-infarct dementia, encephalitis which include encephalitis periaxialis, viral encephalitis such as epidemic encephalitis, Japanese Encephalitis, St. Louis Encephalitis, tick-borne encephalitis and West Nile Fever, acute disseminated encephalomyelitis, meningoencephalitis such as uveomeningoencephalitic syndrome, Postencephalitic Parkinson Disease and subacute sclerosing panencephalitis, encephalomalacia such as periventricular leukomalacia, epilepsy such as generalized epilepsy which includes infantile spasms, absence epilepsy, myoclonic epilepsy which includes MERRF Syndrome, tonic-clonic epilepsy, partial epilepsy such as complex partial epilepsy, frontal lobe epilepsy and temporal lobe epilepsy, post-traumatic epilepsy, status epilepticus such as Epilepsia Partialis Continua, and Hallervorden-Spatz Syndrome.

[0783] Additional neurologic diseases which can be treated or detected with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include hydrocephalus such as Dandy-Walker Syndrome and normal pressure hydrocephalus, hypothalamic diseases such as hypothalamic neoplasms, cerebral malaria, narcolepsy which includes cataplexy, bulbar poliomyelitis, cerebri pseudotumor, Rett Syndrome, Reye's Syndrome, thalamic diseases, cerebral toxoplasmosis, intracranial tuberculoma and Zellweger Syndrome, central nervous system infections such as AIDS Dementia Complex, Brain Abscess, subdural empyema, encephalomyelitis such as Equine Encephalomyelitis, Venezuelan Equine Encephalomyelitis, Necrotizing Hemorrhagic Encephalomyelitis, Visna, and cerebral malaria.

[0784] Additional neurologic diseases which can be treated or detected with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include meningitis such as arachnoiditis, aseptic meningtitis such as viral meningtitis which includes lymphocytic choriomeningitis, Bacterial meningtitis which includes Haemophilus Meningtitis, Listeria Meningtitis, Meningococcal Meningtitis such as Waterhouse-Friderichsen Syndrome, Pneumococcal Meningtitis and meningeal tuberculosis, fungal meningitis such as Cryptococcal Meningtitis, subdural effusion, meningoencephalitis such as uvemeningoencephalitic syndrome, myelitis such as transverse myelitis, neurosyphilis such as tabes dorsalis, poliomyelitis which includes bulbar poliomyelitis and postpoliomyelitis syndrome, prion diseases (such as Creutzfeldt-Jakob Syndrome, Bovine Spongiform Encephalopathy, Gerstmann-Straussler Syndrome, Kuru, Scrapie), and cerebral

toxoplasmosis.

[0785] Additional neurologic diseases which can be treated or detected with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include central nervous system neoplasms such as brain neoplasms that include cerebellar neoplasms such as infratentorial neoplasms, cerebral ventricle neoplasms such as choroid plexus neoplasms, hypothalamic neoplasms and supratentorial neoplasms, meningeal neoplasms, spinal cord neoplasms which include epidural neoplasms, demyelinating diseases such as Canavan Diseases, diffuse cerebral sceloris which includes adrenoleukodystrophy, encephalitis periaxialis, globoid cell leukodystrophy, diffuse cerebral sclerosis such as metachromatic leukodystrophy, allergic encephalomyelitis, necrotizing hemorrhagic encephalomyelitis, progressive multifocal leukoencephalopathy, multiple sclerosis, central pontine myelinolysis, transverse myelitis, neuromyelitis optica, Scrapie, Swayback, Chronic Fatigue Syndrome, Visna, High Pressure Nervous Syndrome, Meningism, spinal cord diseases such as amyotonia congenita, amyotrophic lateral sclerosis, spinal muscular atrophy such as Werdnig-Hoffmann Disease, spinal cord compression, spinal cord neoplasms such as epidural neoplasms, syringomyelia, Tabes Dorsalis, Stiff-Man Syndrome, mental retardation such as Angelman Syndrome, Cri-du-Chat Syndrome, De Lange's Syndrome, Down Syndrome, Gangliosidoses such as gangliosidoses G(M1), Sandhoff Disease, Tay-Sachs Disease, Hartnup Disease, homocystinuria, Laurence-Moon- Biedl Syndrome, Lesch-Nyhan Syndrome, Maple Syrup Urine Disease, mucolipidosis such as fucosidosis, neuronal ceroid-lipofuscinosis, oculocerebrorenal syndrome, phenylketonuria such as maternal phenylketonuria, Prader-Willi Syndrome, Rett Syndrome, Rubinstein-Taybi Syndrome, Tuberous Sclerosis, WAGR Syndrome, nervous system abnormalities such as holoprosencephaly, neural tube defects such as anencephaly which includes hydrangencephaly, Arnold-Chairi Deformity, encephalocele, meningocele, meningomyelocele, spinal dysraphism such as spina bifida cystica and spina bifida occulta.

[0786] Additional neurologic diseases which can be treated or detected with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include hereditary motor and sensory neuropathies which include Charcot-Marie Disease, Hereditary optic atrophy, Refsum's Disease, hereditary spastic paraplegia, Werdnig-Hoffmann Disease, Hereditary Sensory and Autonomic Neuropathies such as Congenital Analgesia and Familial Dysautonomia; Neurologic manifestations (such as agnosia that include Gerstmann's Syndrome, Amnesia such as retrograde amnesia, apraxia, neurogenic

bladder, cataplexy, communicative disorders such as hearing disorders that includes deafness, partial hearing loss, loudness recruitment and tinnitus, language disorders such as aphasia which include agraphia, anomia, broca aphasia, and Wernicke Aphasia, Dyslexia such as Acquired Dyslexia, language development disorders, speech disorders such as aphasia which includes anomia, broca aphasia and Wernicke Aphasia, articulation disorders, communicative disorders such as speech disorders which include dysarthria, echolalia, mutism and stuttering, voice disorders such as aphonia and hoarseness, decerebrate state, delirium, fasciculation, hallucinations, meningism, movement disorders such as angelman syndrome, ataxia, athetosis, chorea, dystonia, hypokinesia, muscle hypotonia, myoclonus, tic, torticollis and tremor, muscle hypertonia such as muscle rigidity such as stiff-man syndrome, muscle spasticity, paralysis such as facial paralysis which includes Herpes Zoster Oticus, Gastroparesis, Hemiplegia, ophthalmoplegia such as diplopia, Duane's Syndrome, Horner's Syndrome, Chronic progressive external ophthalmoplegia such as Kearns Syndrome, Bulbar Paralysis, Tropical Spastic Paraparesis, Paraplegia such as Brown-Sequard Syndrome, quadriplegia, respiratory paralysis and vocal cord paralysis, paresis, phantom limb, taste disorders such as ageusia and dysgeusia, vision disorders such as amblyopia, blindness, color vision defects, diplopia, hemianopsia, scotoma and subnormal vision, sleep disorders such as hypersomnia which includes Kleine-Levin Syndrome, insomnia, and somnambulism, spasm such as trismus, unconsciousness such as coma, persistent vegetative state and syncope and vertigo, neuromuscular diseases such as amyotonia congenita, amyotrophic lateral sclerosis, Lambert-Eaton Myasthenic Syndrome, motor neuron disease, muscular atrophy such as spinal muscular atrophy, Charcot-Marie Disease and Werdnig-Hoffmann Disease, Postpoliomyelitis Syndrome, Muscular Dystrophy, Myasthenia Gravis, Myotonia Atrophica, Myotonia Confenita, Nemaline Myopathy, Familial Periodic Paralysis, Multiplex Paramyloclonus, Tropical Spastic Paraparesis and Stiff-Man Syndrome, peripheral nervous system diseases such as acrodynia, amyloid neuropathies, autonomic nervous system diseases such as Adie's Syndrome, Barre-Lieou Syndrome, Familial Dysautonomia, Horner's Syndrome, Reflex Sympathetic Dystrophy and Shy-Drager Syndrome, Cranial Nerve Diseases such as Acoustic Nerve Diseases such as Acoustic Neuroma which includes Neurofibromatosis 2, Facial Nerve Diseases such as Facial Neuralgia,Melkersson-Rosenthal Syndrome, ocular motility disorders which includes amblyopia, nystagmus, oculomotor nerve paralysis, ophthalmoplegia such as Duane's Syndrome, Horner's Syndrome, Chronic Progressive External Ophthalmoplegia which includes Kearns Syndrome, Strabismus such as

Esotropia and Exotropia, Oculomotor Nerve Paralysis, Optic Nerve Diseases such as Optic Atrophy which includes Hereditary Optic Atrophy, Optic Disk Drusen, Optic Neuritis such as Neuromyelitis Optica, Papilledema, Trigeminal Neuralgia, Vocal Cord Paralysis, Demyelinating Diseases such as Neuromyelitis Optica and Swayback, and Diabetic neuropathies such as diabetic foot.

[0787] Additional neurologic diseases which can be treated or detected with fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include nerve compression syndromes such as carpal tunnel syndrome, tarsal tunnel syndrome, thoracic outlet syndrome such as cervical rib syndrome, ulnar nerve compression syndrome, neuralgia such as causalgia, cervico-brachial neuralgia, facial neuralgia and trigeminal neuralgia, neuritis such as experimental allergic neuritis, optic neuritis, polyneuritis, polyradiculoneuritis and radiculities such as polyradiculitis, hereditary motor and sensory neuropathies such as Charcot-Marie Disease, Hereditary Optic Atrophy, Refsum's Disease, Hereditary Spastic Paraplegia and Werdnig-Hoffmann Disease, Hereditary Sensory and Autonomic Neuropathies which include Congenital Analgesia and Familial Dysautonomia, POEMS Syndrome, Sciatica, Gustatory Sweating and Tetany).

Endocrine Disorders

[0788] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, may be used to treat, prevent, diagnose, and/or prognose disorders and/or diseases related to hormone imbalance, and/or disorders or diseases of the endocrine system.

[0789] Hormones secreted by the glands of the endocrine system control physical growth, sexual function, metabolism, and other functions. Disorders may be classified in two ways: disturbances in the production of hormones, and the inability of tissues to respond to hormones. The etiology of these hormone imbalance or endocrine system diseases, disorders or conditions may be genetic, somatic, such as cancer and some autoimmune diseases, acquired (e.g., by chemotherapy, injury or toxins), or infectious. Moreover, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention can be used as a marker or detector of a particular disease or disorder related to the endocrine system and/or hormone imbalance.

[0790] Endocrine system and/or hormone imbalance and/or diseases encompass

disorders of uterine motility including, but not limited to: complications with pregnancy and labor (e.g., pre-term labor, post-term pregnancy, spontaneous abortion, and slow or stopped labor); and disorders and/or diseases of the menstrual cycle (e.g., dysmenorrhea and endometriosis).

[0791] Endocrine system and/or hormone imbalance disorders and/or diseases include disorders and/or diseases of the pancreas, such as, for example, diabetes mellitus, diabetes insipidus, congenital pancreatic agenesis, pheochromocytoma--islet cell tumor syndrome; disorders and/or diseases of the adrenal glands such as, for example, Addison's Disease, corticosteroid deficiency, virilizing disease, hirsutism, Cushing's Syndrome, hyperaldosteronism, pheochromocytoma; disorders and/or diseases of the pituitary gland, such as, for example, hyperpituitarism, hypopituitarism, pituitary dwarfism, pituitary adenoma, panhypopituitarism, acromegaly, gigantism; disorders and/or diseases of the thyroid, including but not limited to, hyperthyroidism, hypothyroidism, Plummer's disease, Graves' disease (toxic diffuse goiter), toxic nodular goiter, thyroiditis (Hashimoto's thyroiditis, subacute granulomatous thyroiditis, and silent lymphocytic thyroiditis), Pendred's syndrome, myxedema, cretinism, thyrotoxicosis, thyroid hormone coupling defect, thymic aplasia, Hurthle cell tumours of the thyroid, thyroid cancer, thyroid carcinoma, Medullary thyroid carcinoma; disorders and/or diseases of the parathyroid, such as, for example, hyperparathyroidism, hypoparathyroidism; disorders and/or diseases of the hypothalamus.

[0792] In addition, endocrine system and/or hormone imbalance disorders and/or diseases may also include disorders and/or diseases of the testes or ovaries, including cancer. Other disorders and/or diseases of the testes or ovaries further include, for example, ovarian cancer, polycystic ovary syndrome, Klinefelter's syndrome, vanishing testes syndrome (bilateral anorchia), congenital absence of Leydig's cells, cryptorchidism, Noonan's syndrome, myotonic dystrophy, capillary haemangioma of the testis (benign), neoplasias of the testis and neo-testis.

[0793] Moreover, endocrine system and/or hormone imbalance disorders and/or diseases may also include disorders and/or diseases such as, for example, polyglandular deficiency syndromes, pheochromocytoma, neuroblastoma, multiple Endocrine neoplasia, and disorders and/or cancers of endocrine tissues.

[0794] In another embodiment, albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, may be used to diagnose, prognose, prevent, and/or treat endocrine diseases and/or disorders associated with the

tissue(s) in which the Therapeutic protein corresponding to the Therapeutic protein portion of the albumin protein of the invention is expressed,

Reproductive System Disorders

[0795] The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used for the diagnosis, treatment, or prevention of diseases and/or disorders of the reproductive system. Reproductive system disorders that can be treated by the compositions of the invention, include, but are not limited to, reproductive system injuries, infections, neoplastic disorders, congenital defects, and diseases or disorders which result in infertility, complications with pregnancy, labor, or parturition, and postpartum difficulties.

[0796] Reproductive system disorders and/or diseases include diseases and/or disorders of the testes, including testicular atrophy, testicular feminization, cryptorchism (unilateral and bilateral), anorchia, ectopic testis, epididymitis and orchitis (typically resulting from infections such as, for example, gonorrhea, mumps, tuberculosis, and syphilis), testicular torsion, vasitis nodosa, germ cell tumors (e.g., seminomas, embryonal cell carcinomas, teratocarcinomas, choriocarcinomas, yolk sac tumors, and teratomas), stromal tumors (e.g., Leydig cell tumors), hydrocele, hematocele, varicocele, spermatocele, inguinal hernia, and disorders of sperm production (e.g., immotile cilia syndrome, aspermia, asthenozoospermia, azoospermia, oligospermia, and teratozoospermia).

[0797] Reproductive system disorders also include disorders of the prostate gland, such as acute non-bacterial prostatitis, chronic non-bacterial prostatitis, acute bacterial prostatitis, chronic bacterial prostatitis, prostatodystonia, prostatosis, granulomatous prostatitis, malacoplakia, benign prostatic hypertrophy or hyperplasia, and prostate neoplastic disorders, including adenocarcinomas, transitional cell carcinomas, ductal carcinomas, and squamous cell carcinomas.

[0798] Additionally, the compositions of the invention may be useful in the diagnosis, treatment, and/or prevention of disorders or diseases of the penis and urethra, including inflammatory disorders, such as balanoposthitis, balanitis xerotica obliterans, phimosis, paraphimosis, syphilis, herpes simplex virus, gonorrhea, non-gonococcal urethritis, chlamydia, mycoplasma, trichomonas, HIV, AIDS, Reiter's syndrome, condyloma acuminatum, condyloma latum, and pearly penile papules; urethral abnormalities, such as hypospadias, epispadias, and phimosis; premalignant lesions, including Erythroplasia of

Queyrat, Bowen's disease, Bowenoid paplosis, giant condyloma of Buscke-Lowenstein, and varrucous carcinoma; penile cancers, including squamous cell carcinomas, carcinoma in situ, verrucous carcinoma, and disseminated penile carcinoma; urethral neoplastic disorders, including penile urethral carcinoma, bulbomembranous urethral carcinoma, and prostatic urethral carcinoma; and erectile disorders, such as priapism, Peyronie's disease, erectile dysfunction, and impotence.

[0799] Moreover, diseases and/or disorders of the vas deferens include vasculititis and CBAVD (congenital bilateral absence of the vas deferens); additionally, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used in the diagnosis, treatment, and/or prevention of diseases and/or disorders of the seminal vesicles, including hydatid disease, congenital chloride diarrhea, and polycystic kidney disease.

[0800] Other disorders and/or diseases of the male reproductive system include, for example, Klinefelter's syndrome, Young's syndrome, premature ejaculation, diabetes mellitus, cystic fibrosis, Kartagener's syndrome, high fever, multiple sclerosis, and gynecomastia.

[0801] Further, the polynucleotides, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used in the diagnosis, treatment, and/or prevention of diseases and/or disorders of the vagina and vulva, including bacterial vaginosis, candida vaginitis, herpes simplex virus, chancroid, granuloma inguinale, lymphogranuloma venereum, scabies, human papillomavirus, vaginal trauma, vulvar trauma, adenosis, chlamydia vaginitis, gonorrhea, trichomonas vaginitis, condyloma acuminatum, syphilis, molluscum contagiosum, atrophic vaginitis, Paget's disease, lichen sclerosus, lichen planus, vulvodynia, toxic shock syndrome, vaginismus, vulvovaginitis, vulvar vestibulitis, and neoplastic disorders, such as squamous cell hyperplasia, clear cell carcinoma, basal cell carcinoma, melanomas, cancer of Bartholin's gland, and vulvar intraepithelial neoplasia.

[0802] Disorders and/or diseases of the uterus include dysmenorrhea, retroverted uterus, endometriosis, fibroids, adenomyosis, anovulatory bleeding, amenorrhea, Cushing's syndrome, hydatidiform moles, Asherman's syndrome, premature menopause, precocious puberty, uterine polyps, dysfunctional uterine bleeding (e.g., due to aberrant hormonal signals), and neoplastic disorders, such as adenocarcinomas, keiomyosarcomas, and sarcomas. Additionally, the albumin fusion proteins of the invention and/or polynucleotides

encoding albumin fusion proteins of the invention may be useful as a marker or detector of, as well as in the diagnosis, treatment, and/or prevention of congenital uterine abnormalities, such as bicornuate uterus, septate uterus, simple unicornuate uterus, unicornuate uterus with a noncavitary rudimentary horn, unicornuate uterus with a non-communicating cavitary rudimentary horn, unicornuate uterus with a communicating cavitary horn, arcuate uterus, uterine didelfus, and T-shaped uterus.

[0803] Ovarian diseases and/or disorders include anovulation, polycystic ovary syndrome (Stein-Leventhal syndrome), ovarian cysts, ovarian hypofunction, ovarian insensitivity to gonadotropins, ovarian overproduction of androgens, right ovarian vein syndrome, amenorrhea, hirutism, and ovarian cancer (including, but not limited to, primary and secondary cancerous growth, Sertoli-Leydig tumors, endometriod carcinoma of the ovary, ovarian papillary serous adenocarcinoma, ovarian mucinous adenocarcinoma, and Ovarian Krukenberg tumors).

[0804] Cervical diseases and/or disorders include cervicitis, chronic cervicitis, mucopurulent cervicitis, cervical dysplasia, cervical polyps, Nabothian cysts, cervical erosion, cervical incompetence, and cervical neoplasms (including, for example, cervical carcinoma, squamous metaplasia, squamous cell carcinoma, adenosquamous cell neoplasia, and columnar cell neoplasia).

[0805] Additionally, diseases and/or disorders of the reproductive system include disorders and/or diseases of pregnancy, including miscarriage and stillbirth, such as early abortion, late abortion, spontaneous abortion, induced abortion, therapeutic abortion, threatened abortion, missed abortion, incomplete abortion, complete abortion, habitual abortion, missed abortion, and septic abortion; ectopic pregnancy, anemia, Rh incompatibility, vaginal bleeding during pregnancy, gestational diabetes, intrauterine growth retardation, polyhydramnios, HELLP syndrome, abruptio placentae, placenta previa, hyperemesis, preeclampsia, eclampsia, herpes gestationis, and urticaria of pregnancy. Additionally, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may be used in the diagnosis, treatment, and/or prevention of diseases that can complicate pregnancy, including heart disease, heart failure, rheumatic heart disease, congenital heart disease, mitral valve prolapse, high blood pressure, anemia, kidney disease, infectious disease (e.g., rubella, cytomegalovirus, toxoplasmosis, infectious hepatitis, chlamydia, HIV, AIDS, and genital herpes), diabetes mellitus, Graves' disease, thyroiditis, hypothyroidism, Hashimoto's thyroiditis, chronic active hepatitis,

cirrhosis of the liver, primary biliary cirrhosis, asthma, systemic lupus eryematosis, rheumatoid arthritis, myasthenia gravis, idiopathic thrombocytopenic purpura, appendicitis, ovarian cysts, gallbladder disorders,and obstruction of the intestine.

[0806] Complications associated with labor and parturition include premature rupture of the membranes, pre-term labor, post-term pregnancy, postmaturity, labor that progresses too slowly, fetal distress (e.g., abnormal heart rate (fetal or maternal), breathing problems, and abnormal fetal position), shoulder dystocia, prolapsed umbilical cord, amniotic fluid embolism, and aberrant uterine bleeding.

[0807] Further, diseases and/or disorders of the postdelivery period, including endometritis, myometritis, parametritis, peritonitis, pelvic thrombophlebitis, pulmonary embolism, endotoxemia, pyelonephritis, saphenous thrombophlebitis, mastitis, cystitis, postpartum hemorrhage, and inverted uterus.

[0808] Other disorders and/or diseases of the female reproductive system that may be diagnosed, treated, and/or prevented by the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, for example, Turner's syndrome, pseudohermaphroditism, premenstrual syndrome, pelvic inflammatory disease, pelvic congestion (vascular engorgement), frigidity, anorgasmia, dyspareunia, ruptured fallopian tube, and Mittelschmerz.

Infectious Disease

[0809] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention can be used to treat or detect infectious agents. For example, by increasing the immune response, particularly increasing the proliferation and differentiation of B and/or T cells, infectious diseases may be treated. The immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may also directly inhibit the infectious agent, without necessarily eliciting an immune response.

[0810] Viruses are one example of an infectious agent that can cause disease or symptoms that can be treated or detected by albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention. Examples of viruses, include, but are not limited to Examples of viruses, include, but are not limited to the

following DNA and RNA viruses and viral families: Arbovirus, Adenoviridae, Arenaviridae, Arterivirus, Birnaviridae, Bunyaviridae, Caliciviridae, Circoviridae, Coronaviridae, Dengue, EBV, HIV, Flaviviridae, Hepadnaviridae (Hepatitis), Herpesviridae (such as, Cytomegalovirus, Herpes Simplex, Herpes Zoster), Mononegavirus (e.g., Paramyxoviridae, Morbillivirus, Rhabdoviridae), Orthomyxoviridae (e.g., Influenza A, Influenza B, and parainfluenza), Papiloma virus, Papovaviridae, Parvoviridae, Picornaviridae, Poxviridae (such as Smallpox or Vaccinia), Reoviridae (e.g., Rotavirus), Retroviridae (HTLV-I, HTLV-II, Lentivirus), and Togaviridae (e.g., Rubivirus). Viruses falling within these families can cause a variety of diseases or symptoms, including, but not limited to: arthritis, bronchiollitis, respiratory syncytial virus, encephalitis, eye infections (e.g., conjunctivitis, keratitis), chronic fatigue syndrome, hepatitis (A, B, C, E, Chronic Active, Delta), Japanese B encephalitis, Junin, Chikungunya, Rift Valley fever, yellow fever, meningitis, opportunistic infections (e.g., AIDS), pneumonia, Burkitt's Lymphoma, chickenpox, hemorrhagic fever, Measles, Mumps, Parainfluenza, Rabies, the common cold, Polio, leukemia, Rubella, sexually transmitted diseases, skin diseases (e.g., Kaposi's, warts), and viremia. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, can be used to treat or detect any of these symptoms or diseases. In specific embodiments, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to treat: meningitis, Dengue, EBV, and/or hepatitis (e.g., hepatitis B). In an additional specific embodiment fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to treat patients nonresponsive to one or more other commercially available hepatitis vaccines. In a further specific embodiment fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to treat AIDS.

[0811] Similarly, bacterial and fungal agents that can cause disease or symptoms and that can be treated or detected by albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention include, but not limited to, the following Gram-Negative and Gram-positive bacteria, bacterial families, and fungi: Actinomyces (e.g., Norcardia), Acinetobacter, *Cryptococcus neoformans*, Aspergillus, Bacillaceae (e.g., *Bacillus anthrasis*), Bacteroides (e.g., *Bacteroides fragilis*), Blastomycosis, Bordetella, Borrelia (e.g., *Borrelia burgdorferi*), Brucella, Candidia, Campylobacter, Chlamydia, Clostridium (e.g., *Clostridium botulinum*, *Clostridium dificile*, *Clostridium perfringens*, *Clostridium tetani*), Coccidioides, Corynebacterium (e.g., *Corynebacterium*

diptheriae), Cryptococcus, Dermatocycoses, *E. coli* (e.g., Enterotoxigenic *E. coli* and Enterohemorrhagic *E. coli*), Enterobacter (e.g. *Enterobacter aerogenes*), Enterobacteriaceae (Klebsiella, Salmonella (e.g., *Salmonella typhi*, *Salmonella enteritidis, Salmonella typhi*), Serratia, Yersinia, Shigella), Erysipelothrix, Haemophilus (e.g., *Haemophilus influenza* type B), Helicobacter, Legionella (e.g., *Legionella pneumophila*), Leptospira, Listeria (e.g., *Listeria monocytogenes*), Mycoplasma, Mycobacterium (e.g., *Mycobacterium leprae and Mycobacterium tuberculosis)*, Vibrio (e.g., *Vibrio cholerae)*, Neisseriaceae (e.g., *Neisseria gonorrhea, Neisseria meningitidis*), Pasteurellacea, Proteus, Pseudomonas (e.g., *Pseudomonas aeruginosa*), Rickettsiaceae, Spirochetes (e.g., Treponema spp., Leptospira spp., Borrelia spp.), Shigella spp., Staphylococcus (e.g., *Staphylococcus aureus*), Meningiococcus, Pneumococcus and Streptococcus (e.g., *Streptococcus pneumoniae* and Groups A, B, and C Streptococci), and Ureaplasmas. These bacterial, parasitic, and fungal families can cause diseases or symptoms, including, but not limited to: antibiotic-resistant infections, bacteremia, endocarditis, septicemia, eye infections (e.g., conjunctivitis), uveitis, tuberculosis, gingivitis, bacterial diarrhea, opportunistic infections (e.g., AIDS related infections), paronychia, prosthesis-related infections, dental caries, Reiter's Disease, respiratory tract infections, such as Whooping Cough or Empyema, sepsis, Lyme Disease, Cat-Scratch Disease, dysentery, paratyphoid fever, food poisoning, Legionella disease, chronic and acute inflammation, erythema, yeast infections, typhoid, pneumonia, gonorrhea, meningitis (e.g., mengitis types A and B), chlamydia, syphillis, diphtheria, leprosy, brucellosis, peptic ulcers, anthrax, spontaneous abortions, birth defects, pneumonia, lung infections, ear infections, deafness, blindness, lethargy, malaise, vomiting, chronic diarrhea, Crohn's disease, colitis, vaginosis, sterility, pelvic inflammatory diseases, candidiasis, paratuberculosis, tuberculosis, lupus, botulism, gangrene, tetanus, impetigo, Rheumatic Fever, Scarlet Fever, sexually transmitted diseases, skin diseases (e.g., cellulitis, dermatocycoses), toxemia, urinary tract infections, wound infections, noscomial infections. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, can be used to treat or detect any of these symptoms or diseases. In specific embodiments, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to treat: tetanus, diptheria, botulism, and/or meningitis type B.

[0812] Moreover, parasitic agents causing disease or symptoms that can be treated, prevented, and/or diagnosed by fusion proteins of the invention and/or polynucleotides

encoding albumin fusion proteins of the invention include, but not limited to, the following families or class: Amebiasis, Babesiosis, Coccidiosis, Cryptosporidiosis, Dientamoebiasis, Dourine, Ectoparasitic, Giardias, Helminthiasis, Leishmaniasis, Schistisoma, Theileriasis, Toxoplasmosis, Trypanosomiasis, and Trichomonas and Sporozoans (e.g., *Plasmodium virax*, *Plasmodium falciparium*, *Plasmodium malariae* and *Plasmodium ovale*). These parasites can cause a variety of diseases or symptoms, including, but not limited to: Scabies, Trombiculiasis, eye infections, intestinal disease (e.g., dysentery, giardiasis), liver disease, lung disease, opportunistic infections (e.g., AIDS related), malaria, pregnancy complications, and toxoplasmosis. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, can be used to treat, prevent, and/or diagnose any of these symptoms or diseases. In specific embodiments, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention are used to treat, prevent, and/or diagnose malaria.

[0813] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention could either be by administering an effective amount of an albumin fusion protein of the invention to the patient, or by removing cells from the patient, supplying the cells with a polynucleotide of the present invention, and returning the engineered cells to the patient (ex vivo therapy). Moreover, the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention can be used as an antigen in a vaccine to raise an immune response against infectious disease.

Regeneration

[0814] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention can be used to differentiate, proliferate, and attract cells, leading to the regeneration of tissues. (See, Science 276:59-87 (1997)). The regeneration of tissues could be used to repair, replace, or protect tissue damaged by congenital defects, trauma (wounds, burns, incisions, or ulcers), age, disease (e.g. osteoporosis, osteocarthritis, periodontal disease, liver failure), surgery, including cosmetic plastic surgery, fibrosis, reperfusion injury, or systemic cytokine damage.

[0815] Tissues that could be regenerated using the present invention include organs (e.g., pancreas, liver, intestine, kidney, skin, endothelium), muscle (smooth, skeletal or cardiac), vasculature (including vascular and lymphatics), nervous, hematopoietic, and skeletal (bone, cartilage, tendon, and ligament) tissue. Preferably, regeneration occurs

without or decreased scarring. Regeneration also may include angiogenesis.

[0816] Moreover, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, may increase regeneration of tissues difficult to heal. For example, increased tendon/ligament regeneration would quicken recovery time after damage. Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention could also be used prophylactically in an effort to avoid damage. Specific diseases that could be treated include of tendinitis, carpal tunnel syndrome, and other tendon or ligament defects. A further example of tissue regeneration of non-healing wounds includes pressure ulcers, ulcers associated with vascular insufficiency, surgical, and traumatic wounds.

[0817] Similarly, nerve and brain tissue could also be regenerated by using fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, to proliferate and differentiate nerve cells. Diseases that could be treated using this method include central and peripheral nervous system diseases, neuropathies, or mechanical and traumatic disorders (e.g., spinal cord disorders, head trauma, cerebrovascular disease, and stoke). Specifically, diseases associated with peripheral nerve injuries, peripheral neuropathy (e.g., resulting from chemotherapy or other medical therapies), localized neuropathies, and central nervous system diseases (e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome), could all be treated using the albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention.

Gastrointestinal Disorders

[0818] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention, may be used to treat, prevent, diagnose, and/or prognose gastrointestinal disorders, including inflammatory diseases and/or conditions, infections, cancers (e.g., intestinal neoplasms (carcinoid tumor of the small intestine, non-Hodgkin's lymphoma of the small intestine, small bowl lymphoma)), and ulcers, such as peptic ulcers.

[0819] Gastrointestinal disorders include dysphagia, odynophagia, inflammation of the esophagus, peptic esophagitis, gastric reflux, submucosal fibrosis and stricturing, Mallory-Weiss lesions, leiomyomas, lipomas, epidermal cancers, adeoncarcinomas, gastric

retention disorders, gastroenteritis, gastric atrophy, gastric/stomach cancers, polyps of the stomach, autoimmune disorders such as pernicious anemia, pyloric stenosis, gastritis (bacterial, viral, eosinophilic, stress-induced, chronic erosive, atrophic, plasma cell, and Ménétrier's), and peritoneal diseases (e.g., chyloperioneum, hemoperitoneum, mesenteric cyst, mesenteric lymphadenitis, mesenteric vascular occlusion, panniculitis, neoplasms, peritonitis, pneumoperitoneum, bubphrenic abscess,).

[0820] Gastrointestinal disorders also include disorders associated with the small intestine, such as malabsorption syndromes, distension, irritable bowel syndrome, sugar intolerance, celiac disease, duodenal ulcers, duodenitis, tropical sprue, Whipple's disease, intestinal lymphangiectasia, Crohn's disease, appendicitis, obstructions of the ileum, Meckel's diverticulum, multiple diverticula, failure of complete rotation of the small and large intestine, lymphoma, and bacterial and parasitic diseases (such as Traveler's diarrhea, typhoid and paratyphoid, cholera, infection by Roundworms (*Ascariasis lumbricoides*), Hookworms (*Ancylostoma duodenale*), Threadworms (*Enterobius vermicularis*), Tapeworms (*Taenia saginata*, *Echinococcus granulosus*, *Diphyllobothrium spp.*, and *T. solium*).

[0821] Liver diseases and/or disorders include intrahepatic cholestasis (alagille syndrome, biliary liver cirrhosis), fatty liver (alcoholic fatty liver, reye syndrome), hepatic vein thrombosis, hepatolentricular degeneration, hepatomegaly, hepatopulmonary syndrome, hepatorenal syndrome, portal hypertension (esophageal and gastric varices), liver abscess (amebic liver abscess), liver cirrhosis (alcoholic, biliary and experimental), alcoholic liver diseases (fatty liver, hepatitis, cirrhosis), parasitic (hepatic echinococcosis, fascioliasis, amebic liver abscess), jaundice (hemolytic, hepatocellular, and cholestatic), cholestasis, portal hypertension, liver enlargement, ascites, hepatitis (alcoholic hepatitis, animal hepatitis, chronic hepatitis (autoimmune, hepatitis B, hepatitis C, hepatitis D, drug induced), toxic hepatitis, viral human hepatitis (hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatitis E), Wilson's disease, granulomatous hepatitis, secondary biliary cirrhosis, hepatic encephalopathy, portal hypertension, varices, hepatic encephalopathy, primary biliary cirrhosis, primary sclerosing cholangitis, hepatocellular adenoma, hemangiomas, bile stones, liver failure (hepatic encephalopathy, acute liver failure), and liver neoplasms (angiomyolipoma, calcified liver metastases, cystic liver metastases, epithelial tumors, fibrolamellar hepatocarcinoma, focal nodular hyperplasia, hepatic adenoma, hepatobiliary cystadenoma, hepatoblastoma, hepatocellular carcinoma, hepatoma, liver cancer, liver hemangioendothelioma, mesenchymal hamartoma, mesenchymal tumors of liver, nodular

regenerative hyperplasia, benign liver tumors (Hepatic cysts [Simple cysts, Polycystic liver disease, Hepatobiliary cystadenoma, Choledochal cyst], Mesenchymal tumors [Mesenchymal hamartoma, Infantile hemangioendothelioma, Hemangioma, Peliosis hepatis, Lipomas, Inflammatory pseudotumor, Miscellaneous], Epithelial tumors [Bile duct epithelium (Bile duct hamartoma, Bile duct adenoma), Hepatocyte (Adenoma, Focal nodular hyperplasia, Nodular regenerative hyperplasia)], malignant liver tumors [hepatocellular, hepatoblastoma, hepatocellular carcinoma, cholangiocellular, cholangiocarcinoma, cystadenocarcinoma, tumors of blood vessels, angiosarcoma, Karposi's sarcoma, hemangioendothelioma, other tumors, embryonal sarcoma, fibrosarcoma, leiomyosarcoma, rhabdomyosarcoma, carcinosarcoma, teratoma, carcinoid, squamous carcinoma, primary lymphoma]), peliosis hepatis, erythrohepatic porphyria, hepatic porphyria (acute intermittent porphyria, porphyria cutanea tarda), Zellweger syndrome).

[0822] Pancreatic diseases and/or disorders include acute pancreatitis, chronic pancreatitis (acute necrotizing pancreatitis, alcoholic pancreatitis), neoplasms (adenocarcinoma of the pancreas, cystadenocarcinoma, insulinoma, gastrinoma, and glucagonoma, cystic neoplasms, islet-cell tumors, pancreoblastoma), and other pancreatic diseases (e.g., cystic fibrosis, cyst (pancreatic pseudocyst, pancreatic fistula, insufficiency)).

[0823] Gallbladder diseases include gallstones (cholelithiasis and choledocholithiasis), postcholecystectomy syndrome, diverticulosis of the gallbladder, acute cholecystitis, chronic cholecystitis, bile duct tumors, and mucocele.

[0824] Diseases and/or disorders of the large intestine include antibiotic-associated colitis, diverticulitis, ulcerative colitis, acquired megacolon, abscesses, fungal and bacterial infections, anorectal disorders (e.g., fissures, hemorrhoids), colonic diseases (colitis, colonic neoplasms [colon cancer, adenomatous colon polyps (e.g., villous adenoma), colon carcinoma, colorectal cancer], colonic diverticulitis, colonic diverticulosis, megacolon [Hirschsprung disease, toxic megacolon]; sigmoid diseases [proctocolitis, sigmoin neoplasms]), constipation, Crohn's disease, diarrhea (infantile diarrhea, dysentery), duodenal diseases (duodenal neoplasms, duodenal obstruction, duodenal ulcer, duodenitis), enteritis (enterocolitis), HIV enteropathy, ileal diseases (ileal neoplasms, ileitis), immunoproliferative small intestinal disease, inflammatory bowel disease (ulcerative colitis, Crohn's disease), intestinal atresia, parasitic diseases (anisakiasis, balantidiasis, blastocystis infections, cryptosporidiosis, dientamoebiasis, amebic dysentery, giardiasis), intestinal fistula (rectal fistula), intestinal neoplasms (cecal neoplasms, colonic neoplasms, duodenal neoplasms, ileal

neoplasms, intestinal polyps, jejunal neoplasms, rectal neoplasms), intestinal obstruction (afferent loop syndrome, duodenal obstruction, impacted feces, intestinal pseudo-obstruction [cecal volvulus], intussusception), intestinal perforation, intestinal polyps (colonic polyps, gardner syndrome, peutz-jeghers syndrome), jejunal diseases (jejunal neoplasms), malabsorption syndromes (blind loop syndrome, celiac disease, lactose intolerance, short bowl syndrome, tropical sprue, whipple's disease), mesenteric vascular occlusion, pneumatosis cystoides intestinalis, protein-losing enteropathies (intestinal lymphagiectasis), rectal diseases (anus diseases, fecal incontinence, hemorrhoids, proctitis, rectal fistula, rectal prolapse, rectocele), peptic ulcer (duodenal ulcer, peptic esophagitis, hemorrhage, perforation, stomach ulcer, Zollinger-Ellison syndrome), postgastrectomy syndromes (dumping syndrome), stomach diseases (e.g., achlorhydria, duodenogastric reflux (bile reflux), gastric antral vascular ectasia, gastric fistula, gastric outlet obstruction, gastritis (atrophic or hypertrophic), gastroparesis, stomach dilatation, stomach diverticulum, stomach neoplasms (gastric cancer, gastric polyps, gastric adenocarcinoma, hyperplastic gastric polyp), stomach rupture, stomach ulcer, stomach volvulus), tuberculosis, visceroptosis, vomiting (e.g., hematemesis, hyperemesis gravidarum, postoperative nausea and vomiting) and hemorrhagic colitis.

[0825] Further diseases and/or disorders of the gastrointestinal system include biliary tract diseases, such as, gastroschisis, fistula (e.g., biliary fistula, esophageal fistula, gastric fistula, intestinal fistula, pancreatic fistula), neoplasms (e.g., biliary tract neoplasms, esophageal neoplasms, such as adenocarcinoma of the esophagus, esophageal squamous cell carcinoma, gastrointestinal neoplasms, pancreatic neoplasms, such as adenocarcinoma of the pancreas, mucinous cystic neoplasm of the pancreas, pancreatic cystic neoplasms, pancreatoblastoma, and peritoneal neoplasms), esophageal disease (e.g., bullous diseases, candidiasis, glycogenic acanthosis, ulceration, barrett esophagus varices, atresia, cyst, diverticulum (e.g., Zenker's diverticulum), fistula (e.g., tracheoesophageal fistula), motility disorders (e.g., CREST syndrome, deglutition disorders, achalasia, spasm, gastroesophageal reflux), neoplasms, perforation (e.g., Boerhaave syndrome, Mallory-Weiss syndrome), stenosis, esophagitis, diaphragmatic hernia (e.g., hiatal hernia); gastrointestinal diseases, such as, gastroenteritis (e.g., cholera morbus, norwalk virus infection), hemorrhage (e.g., hematemesis, melena, peptic ulcer hemorrhage), stomach neoplasms (gastric cancer, gastric polyps, gastric adenocarcinoma, stomach cancer)), hernia (e.g., congenital diaphragmatic hernia, femoral hernia, inguinal hernia, obturator hernia, umbilical hernia, ventral hernia), and

intestinal diseases (e.g., cecal diseases (appendicitis, cecal neoplasms)).

Chemotaxis

[0826] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may have chemotaxis activity. A chemotaxic molecule attracts or mobilizes cells (e.g., monocytes, fibroblasts, neutrophils, T-cells, mast cells, eosinophils, epithelial and/or endothelial cells) to a particular site in the body, such as inflammation, infection, or site of hyperproliferation. The mobilized cells can then fight off and/or heal the particular trauma or abnormality.

[0827] Albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may increase chemotaxic activity of particular cells. These chemotactic molecules can then be used to treat inflammation, infection, hyperproliferative disorders, or any immune system disorder by increasing the number of cells targeted to a particular location in the body. For example, chemotaxic molecules can be used to treat wounds and other trauma to tissues by attracting immune cells to the injured location. Chemotactic molecules of the present invention can also attract fibroblasts, which can be used to treat wounds.

[0828] It is also contemplated that fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may inhibit chemotactic activity. These molecules could also be used to treat disorders. Thus, fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention could be used as an inhibitor of chemotaxis.

Binding Activity

[0829] Albumin fusion proteins of the invention may be used to screen for molecules that bind to the Therapeutic protein portion of the fusion protein or for molecules to which the Therapeutic protein portion of the fusion protein binds. The binding of the fusion protein and the molecule may activate (agonist), increase, inhibit (antagonist), or decrease activity of the fusion protein or the molecule bound. Examples of such molecules include antibodies, oligonucleotides, proteins (e.g., receptors), or small molecules.

[0830] Preferably, the molecule is closely related to the natural ligand of the Therapeutic protein portion of the fusion protein of the invention, e.g., a fragment of the

ligand, or a natural substrate, a ligand, a structural or functional mimetic. (See, Coligan et al., Current Protocols in Immunology 1(2):Chapter 5 (1991)). Similarly, the molecule can be closely related to the natural receptor to which the Therapeutic protein portion of an albumin fusion protein of the invention binds, or at least, a fragment of the receptor capable of being bound by the Therapeutic protein portion of an albumin fusion protein of the invention (e.g., active site). In either case, the molecule can be rationally designed using known techniques.

[0831] Preferably, the screening for these molecules involves producing appropriate cells which express the albumin fusion proteins of the invention. Preferred cells include cells from mammals, yeast, Drosophila, or *E. coli*.

[0832] The assay may simply test binding of a candidate compound to an albumin fusion protein of the invention, wherein binding is detected by a label, or in an assay involving competition with a labeled competitor. Further, the assay may test whether the candidate compound results in a signal generated by binding to the fusion protein.

[0833] Alternatively, the assay can be carried out using cell-free preparations, fusion protein/molecule affixed to a solid support, chemical libraries, or natural product mixtures. The assay may also simply comprise the steps of mixing a candidate compound with a solution containing an albumin fusion protein, measuring fusion protein/molecule activity or binding, and comparing the fusion protein/molecule activity or binding to a standard.

[0834] Preferably, an ELISA assay can measure fusion protein level or activity in a sample (e.g., biological sample) using a monoclonal or polyclonal antibody. The antibody can measure fusion protein level or activity by either binding, directly or indirectly, to the albumin fusion protein or by competing with the albumin fusion protein for a substrate.

[0835] Additionally, the receptor to which a Therapeutic protein portion of an albumin fusion protein of the invention binds can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). For example, in cases wherein the Therapeutic protein portion of the fusion protein corresponds to FGF, expression cloning may be employed wherein polyadenylated RNA is prepared from a cell responsive to the albumin fusion protein, for example, NIH3T3 cells which are known to contain multiple receptors for the FGF family proteins, and SC-3 cells, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to the albumin fusion protein. Transfected cells which are grown on glass slides are exposed to the albumin fusion protein of the present invention, after they have been labeled. The albumin

fusion proteins can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase.

[0836] Following fixation and incubation, the slides are subjected to auto-radiographic analysis. Positive pools are identified and sub-pools are prepared and re-transfected using an iterative sub-pooling and re-screening process, eventually yielding a single clones that encodes the putative receptor.

[0837] As an alternative approach for receptor identification, a labeled albumin fusion protein can be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule for the Therapeutoc protein component of an albumin fusion protein of the invention, the linked material may be resolved by PAGE analysis and exposed to X-ray film. The labeled complex containing the receptors of the fusion protein can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the genes encoding the putative receptors.

[0838] Moreover, the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling") may be employed to modulate the activities of the fusion protein, and/or Therapeutic protein portion or albumin component of an albumin fusion protein of the present invention, thereby effectively generating agonists and antagonists of an albumin fusion protein of the present invention. *See generally*, U.S. Patent Nos. 5,605,793, 5,811,238, 5,830,721, 5,834,252, and 5,837,458, and Patten, P. A., *et al., Curr. Opinion Biotechnol.* 8:724-33 (1997); Harayama, S. *Trends Biotechnol.* 16(2):76-82 (1998); Hansson, L. O., *et al., J. Mol. Biol.* 287:265-76 (1999); and Lorenzo, M. M. and Blasco, R. *Biotechniques* 24(2):308-13 (1998); each of these patents and publications are hereby incorporated by reference). In one embodiment, alteration of polynucleotides encoding albumin fusion proteins of the invention and thus, the albumin fusion proteins encoded thereby, may be achieved by DNA shuffling. DNA shuffling involves the assembly of two or more DNA segments into a desired molecule by homologous, or site-specific, recombination. In another embodiment, polynucleotides encoding albumin fusion proteins of the invention and thus, the albumin fusion proteins encoded thereby, may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. In another embodiment, one or more components, motifs, sections, parts, domains, fragments, etc., of an

albumin fusion protein of the present invention may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules. In preferred embodiments, the heterologous molecules are family members. In further preferred embodiments, the heterologous molecule is a growth factor such as, for example, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-I), transforming growth factor (TGF)-alpha, epidermal growth factor (EGF), fibroblast growth factor (FGF), TGF-beta, bone morphogenetic protein (BMP)-2, BMP-4, BMP-5, BMP-6, BMP-7, activins A and B, decapentaplegic(dpp), 60A, OP-2, dorsalin, growth differentiation factors (GDFs), nodal, MIS, inhibin-alpha, TGF-beta1, TGF-beta2, TGF-beta3, TGF-beta5, and glial-derived neurotrophic factor (GDNF).

[0839] Other preferred fragments are biologically active fragments of the Therapeutic protein portion and/or albumin component of the albumin fusion proteins of the present invention. Biologically active fragments are those exhibiting activity similar, but not necessarily identical, to an activity of a Therapeutic protein portion and/or albumin component of the albumin fusion proteins of the present invention. The biological activity of the fragments may include an improved desired activity, or a decreased undesirable activity.

[0840] Additionally, this invention provides a method of screening compounds to identify those which modulate the action of an albumin fusion protein of the present invention. An example of such an assay comprises combining a mammalian fibroblast cell, an albumin fusion protein of the present invention, and the compound to be screened and ^{3}[H] thymidine under cell culture conditions where the fibroblast cell would normally proliferate. A control assay may be performed in the absence of the compound to be screened and compared to the amount of fibroblast proliferation in the presence of the compound to determine if the compound stimulates proliferation by determining the uptake of ^{3}[H] thymidine in each case. The amount of fibroblast cell proliferation is measured by liquid scintillation chromatography which measures the incorporation of ^{3}[H] thymidine. Both agonist and antagonist compounds may be identified by this procedure.

[0841] In another method, a mammalian cell or membrane preparation expressing a receptor for the Therapeutic protien component of a fusion protine of the invention is incubated with a labeled fusion protein of the present invention in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured. Alternatively, the response of a known second messenger system following

interaction of a compound to be screened and the receptor is measured and the ability of the compound to bind to the receptor and elicit a second messenger response is measured to determine if the compound is a potential fusion protein. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis.

[0842] All of these above assays can be used as diagnostic or prognostic markers. The molecules discovered using these assays can be used to treat disease or to bring about a particular result in a patient (e.g., blood vessel growth) by activating or inhibiting the fusion protein/molecule. Moreover, the assays can discover agents which may inhibit or enhance the production of the albumin fusion proteins of the invention from suitably manipulated cells or tissues.

[0843] Therefore, the invention includes a method of identifying compounds which bind to an albumin fusion protein of the invention comprising the steps of: (a) incubating a candidate binding compound with an albumin fusion protein of the present invention; and (b) determining if binding has occurred. Moreover, the invention includes a method of identifying agonists/antagonists comprising the steps of: (a) incubating a candidate compound with an albumin fusion protein of the present invention, (b) assaying a biological activity, and (b) determining if a biological activity of the fusion protein has been altered.

Targeted Delivery

[0844] In another embodiment, the invention provides a method of delivering compositions to targeted cells expressing a receptor for a component of an albumin fusion protein of the invention.

[0845] As discussed herein, fusion proteins of the invention may be associated with heterologous polypeptides, heterologous nucleic acids, toxins, or prodrugs via hydrophobic, hydrophilic, ionic and/or covalent interactions. In one embodiment, the invention provides a method for the specific delivery of compositions of the invention to cells by administering fusion proteins of the invention (including antibodies) that are associated with heterologous polypeptides or nucleic acids. In one example, the invention provides a method for delivering a Therapeutic protein into the targeted cell. In another example, the invention provides a method for delivering a single stranded nucleic acid (e.g., antisense or ribozymes) or double stranded nucleic acid (e.g., DNA that can integrate into the cell's genome or replicate episomally and that can be transcribed) into the targeted cell.

[0846] In another embodiment, the invention provides a method for the specific destruction of cells (e.g., the destruction of tumor cells) by administering an albumin fusion protein of the invention (e.g., polypeptides of the invention or antibodies of the invention) in association with toxins or cytotoxic prodrugs.

[0847] By "toxin" is meant compounds that bind and activate endogenous cytotoxic effector systems, radioisotopes, holotoxins, modified toxins, catalytic subunits of toxins, or any molecules or enzymes not normally present in or on the surface of a cell that under defined conditions cause the cell's death. Toxins that may be used according to the methods of the invention include, but are not limited to, radioisotopes known in the art, compounds such as, for example, antibodies (or complement fixing containing portions thereof) that bind an inherent or induced endogenous cytotoxic effector system, thymidine kinase, endonuclease, RNAse, alpha toxin, ricin, abrin, *Pseudomonas* exotoxin A, diphtheria toxin, saporin, momordin, gelonin, pokeweed antiviral protein, alpha-sarcin and cholera toxin. By "cytotoxic prodrug" is meant a non-toxic compound that is converted by an enzyme, normally present in the cell, into a cytotoxic compound. Cytotoxic prodrugs that may be used according to the methods of the invention include, but are not limited to, glutamyl derivatives of benzoic acid mustard alkylating agent, phosphate derivatives of etoposide or mitomycin C, cytosine arabinoside, daunorubisin, and phenoxyacetamide derivatives of doxorubicin.

Drug Screening

[0848] Further contemplated is the use of the albumin fusion proteins of the present invention, or the polynucleotides encoding these fusion proteins, to screen for molecules which modify the activities of the albumin fusion protein of the present invention or proteins corresponding to the Therapeutic protein portion of the albumin fusion protein. Such a method would include contacting the fusion protein with a selected compound(s) suspected of having antagonist or agonist activity, and assaying the activity of the fusion protein following binding.

[0849] This invention is particularly useful for screening therapeutic compounds by using the albumin fusion proteins of the present invention, or binding fragments thereof, in any of a variety of drug screening techniques. The albumin fusion protein employed in such a test may be affixed to a solid support, expressed on a cell surface, free in solution, or located intracellularly. One method of drug screening utilizes eukaryotic or prokaryotic host cells

which are stably transformed with recombinant nucleic acids expressing the albumin fusion protein. Drugs are screened against such transformed cells or supernatants obtained from culturing such cells, in competitive binding assays. One may measure, for example, the formulation of complexes between the agent being tested and an albumin fusion protein of the present invention.

[0850] Thus, the present invention provides methods of screening for drugs or any other agents which affect activities mediated by the albumin fusion proteins of the present invention. These methods comprise contacting such an agent with an albumin fusion protein of the present invention or a fragment thereof and assaying for the presence of a complex between the agent and the albumin fusion protein or a fragment thereof, by methods well known in the art. In such a competitive binding assay, the agents to screen are typically labeled. Following incubation, free agent is separated from that present in bound form, and the amount of free or uncomplexed label is a measure of the ability of a particular agent to bind to the albumin fusion protein of the present invention.

[0851] Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to an albumin fusion protein of the present invention, and is described in great detail in European Patent Application 84/03564, published on September 13, 1984, which is incorporated herein by reference herein. Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with an albumin fusion protein of the present invention and washed. Bound peptides are then detected by methods well known in the art. Purified albumin fusion protein may be coated directly onto plates for use in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies may be used to capture the peptide and immobilize it on the solid support.

[0852] This invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of binding an albumin fusion protein of the present invention specifically compete with a test compound for binding to the albumin fusion protein or fragments thereof. In this manner, the antibodies are used to detect the presence of any peptide which shares one or more antigenic epitopes with an albumin fusion protein of the invention.

Binding Peptides and Other Molecules

[0853] The invention also encompasses screening methods for identifying polypeptides and nonpolypeptides that bind albumin fusion proteins of the invention, and the binding molecules identified thereby. These binding molecules are useful, for example, as agonists and antagonists of the albumin fusion proteins of the invention. Such agonists and antagonists can be used, in accordance with the invention, in the therapeutic embodiments described in detail, below.

[0854] This method comprises the steps of:

contacting an albumin fusion protein of the invention with a plurality of molecules; and

identifying a molecule that binds the albumin fusion protein.

[0855] The step of contacting the albumin fusion protein of the invention with the plurality of molecules may be effected in a number of ways. For example, one may contemplate immobilizing the albumin fusion protein on a solid support and bringing a solution of the plurality of molecules in contact with the immobilized polypeptides. Such a procedure would be akin to an affinity chromatographic process, with the affinity matrix being comprised of the immobilized albumin fusion protein of the invention. The molecules having a selective affinity for the albumin fusion protein can then be purified by affinity selection. The nature of the solid support, process for attachment of the albumin fusion protein to the solid support, solvent, and conditions of the affinity isolation or selection are largely conventional and well known to those of ordinary skill in the art.

[0856] Alternatively, one may also separate a plurality of polypeptides into substantially separate fractions comprising a subset of or individual polypeptides. For instance, one can separate the plurality of polypeptides by gel electrophoresis, column chromatography, or like method known to those of ordinary skill for the separation of polypeptides. The individual polypeptides can also be produced by a transformed host cell in such a way as to be expressed on or about its outer surface (e.g., a recombinant phage). Individual isolates can then be "probed" by an albumin fusion protein of the invention, optionally in the presence of an inducer should one be required for expression, to determine if any selective affinity interaction takes place between the albumin fusion protein and the individual clone. Prior to contacting the albumin fusion protein with each fraction comprising individual polypeptides, the polypeptides could first be transferred to a solid support for additional convenience. Such a solid support may simply be a piece of filter membrane, such

as one made of nitrocellulose or nylon. In this manner, positive clones could be identified from a collection of transformed host cells of an expression library, which harbor a DNA construct encoding a polypeptide having a selective affinity for an albumin fusion protein of the invention. Furthermore, the amino acid sequence of the polypeptide having a selective affinity for an albumin fusion protein of the invention can be determined directly by conventional means or the coding sequence of the DNA encoding the polypeptide can frequently be determined more conveniently. The primary sequence can then be deduced from the corresponding DNA sequence. If the amino acid sequence is to be determined from the polypeptide itself, one may use microsequencing techniques. The sequencing technique may include mass spectroscopy.

[0857] In certain situations, it may be desirable to wash away any unbound polypeptides from a mixture of an albumin fusion protein of the invention and the plurality of polypeptides prior to attempting to determine or to detect the presence of a selective affinity interaction. Such a wash step may be particularly desirable when the albumin fusion protein of the invention or the plurality of polypeptides are bound to a solid support.

[0858] The plurality of molecules provided according to this method may be provided by way of diversity libraries, such as random or combinatorial peptide or nonpeptide libraries which can be screened for molecules that specifically bind an albumin fusion protein of the invention. Many libraries are known in the art that can be used, e.g., chemically synthesized libraries, recombinant (e.g., phage display libraries), and *in vitro* translation-based libraries. Examples of chemically synthesized libraries are described in Fodor et al., Science 251:767-773 (1991); Houghten et al., Nature 354:84-86 (1991); Lam et al., Nature 354:82-84 (1991); Medynski, Bio/Technology 12:709-710 (1994); Gallop et al., J. Medicinal Chemistry 37(9):1233-1251 (1994); Ohlmeyer et al., Proc. Natl. Acad. Sci. USA 90:10922-10926 (1993); Erb et al., Proc. Natl. Acad. Sci. USA 91:11422-11426 (1994); Houghten et al., Biotechniques 13:412 (1992); Jayawickreme et al., Proc. Natl. Acad. Sci. USA 91:1614-1618 (1994); Salmon et al., Proc. Natl. Acad. Sci. USA 90:11708-11712 (1993); PCT Publication No. WO 93/20242; and Brenner and Lerner, Proc. Natl. Acad. Sci. USA 89:5381-5383 (1992).

[0859] Examples of phage display libraries are described in Scott et al., Science 249:386-390 (1990); Devlin et al., Science, 249:404-406 (1990); Christian et al., 1992, J. Mol. Biol. 227:711-718 1992); Lenstra, J. Immunol. Meth. 152:149-157 (1992); Kay et al., Gene 128:59-65 (1993); and PCT Publication No. WO 94/18318 dated Aug. 18, 1994.

[0860] In vitro translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058 dated Apr. 18, 1991; and Mattheakis et al., Proc. Natl. Acad. Sci. USA 91:9022-9026 (1994).

[0861] By way of examples of nonpeptide libraries, a benzodiazepine library (see e.g., Bunin et al., Proc. Natl. Acad. Sci. USA 91:4708-4712 (1994)) can be adapted for use. Peptoid libraries (Simon et al., Proc. Natl. Acad. Sci. USA 89:9367-9371 (1992)) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (Proc. Natl. Acad. Sci. USA 91:11138-11142 (1994)).

[0862] The variety of non-peptide libraries that are useful in the present invention is great. For example, Ecker and Crooke (Bio/Technology 13:351-360 (1995) list benzodiazepines, hydantoins, piperazinediones, biphenyls, sugar analogs, beta-mercaptoketones, arylacetic acids, acylpiperidines, benzopyrans, cubanes, xanthines, aminimides, and oxazolones as among the chemical species that form the basis of various libraries.

[0863] Non-peptide libraries can be classified broadly into two types: decorated monomers and oligomers. Decorated monomer libraries employ a relatively simple scaffold structure upon which a variety functional groups is added. Often the scaffold will be a molecule with a known useful pharmacological activity. For example, the scaffold might be the benzodiazepine structure.

[0864] Non-peptide oligomer libraries utilize a large number of monomers that are assembled together in ways that create new shapes that depend on the order of the monomers. Among the monomer units that have been used are carbamates, pyrrolinones, and morpholinos. Peptoids, peptide-like oligomers in which the side chain is attached to the alpha amino group rather than the alpha carbon, form the basis of another version of non-peptide oligomer libraries. The first non-peptide oligomer libraries utilized a single type of monomer and thus contained a repeating backbone. Recent libraries have utilized more than one monomer, giving the libraries added flexibility.

[0865] Screening the libraries can be accomplished by any of a variety of commonly known methods. See, e.g., the following references, which disclose screening of peptide libraries: Parmley et al., Adv. Exp. Med. Biol. 251:215-218 (1989); Scott et al,. Science 249:386-390 (1990); Fowlkes et al., BioTechniques 13:422-427 (1992); Oldenburg et al., Proc. Natl. Acad. Sci. USA 89:5393-5397 (1992); Yu et al., Cell 76:933-945 (1994); Staudt

et al., Science 241:577-580 (1988); Bock et al., Nature 355:564-566 (1992); Tuerk et al., Proc. Natl. Acad. Sci. USA 89:6988-6992 (1992); Ellington et al., Nature 355:850-852 (1992); U.S. Pat. No. 5,096,815, U.S. Pat. No. 5,223,409, and U.S. Pat. No. 5,198,346, all to Ladner et al.; Rebar et al., Science 263:671-673 (1993); and PCT Publication No. WO 94/18318.

[0866] In a specific embodiment, screening to identify a molecule that binds an albumin fusion protein of the invention can be carried out by contacting the library members with an albumin fusion protein of the invention immobilized on a solid phase and harvesting those library members that bind to the albumin fusion protein. Examples of such screening methods, termed "panning" techniques are described by way of example in Parmley et al., Gene 73:305-318 (1988); Fowlkes et al., BioTechniques 13:422-427 (1992); PCT Publication No. WO 94/18318; and in references cited herein.

[0867] In another embodiment, the two-hybrid system for selecting interacting proteins in yeast (Fields et al., Nature 340:245-246 (1989); Chien et al., Proc. Natl. Acad. Sci. USA 88:9578-9582 (1991) can be used to identify molecules that specifically bind to polypeptides of the invention.

[0868] Where the binding molecule is a polypeptide, the polypeptide can be conveniently selected from any peptide library, including random peptide libraries, combinatorial peptide libraries, or biased peptide libraries. The term "biased" is used herein to mean that the method of generating the library is manipulated so as to restrict one or more parameters that govern the diversity of the resulting collection of molecules, in this case peptides.

[0869] Thus, a truly random peptide library would generate a collection of peptides in which the probability of finding a particular amino acid at a given position of the peptide is the same for all 20 amino acids. A bias can be introduced into the library, however, by specifying, for example, that a lysine occur every fifth amino acid or that positions 4, 8, and 9 of a decapeptide library be fixed to include only arginine. Clearly, many types of biases can be contemplated, and the present invention is not restricted to any particular bias. Furthermore, the present invention contemplates specific types of peptide libraries, such as phage displayed peptide libraries and those that utilize a DNA construct comprising a lambda phage vector with a DNA insert.

[0870] As mentioned above, in the case of a binding molecule that is a polypeptide, the polypeptide may have about 6 to less than about 60 amino acid residues, preferably about

6 to about 10 amino acid residues, and most preferably, about 6 to about 22 amino acids. In another embodiment, a binding polypeptide has in the range of 15-100 amino acids, or 20-50 amino acids.

[0871] The selected binding polypeptide can be obtained by chemical synthesis or recombinant expression.

Other Activities

[0872] An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention, may be employed in treatment for stimulating re-vascularization of ischemic tissues due to various disease conditions such as thrombosis, arteriosclerosis, and other cardiovascular conditions. The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may also be employed to stimulate angiogenesis and limb regeneration, as discussed above.

[0873] An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also be employed for treating wounds due to injuries, burns, post-operative tissue repair, and ulcers since they are mitogenic to various cells of different origins, such as fibroblast cells and skeletal muscle cells, and therefore, facilitate the repair or replacement of damaged or diseased tissue.

[0874] An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also be employed stimulate neuronal growth and to treat and prevent neuronal damage which occurs in certain neuronal disorders or neuro-degenerative conditions such as Alzheimer's disease, Parkinson's disease, and AIDS-related complex. An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may have the ability to stimulate chondrocyte growth, therefore, they may be employed to enhance bone and periodontal regeneration and aid in tissue transplants or bone grafts.

[0875] An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may be also be employed to prevent skin aging due to sunburn by stimulating keratinocyte growth.

[0876] An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also be employed for preventing hair loss, since FGF family members activate hair-forming cells and promotes melanocyte growth. Along the same lines, an albumin fusion protein of the invention and/or polynucleotide encoding an

albumin fusion protein of the invention may be employed to stimulate growth and differentiation of hematopoietic cells and bone marrow cells when used in combination with other cytokines.

[0877] An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also be employed to maintain organs before transplantation or for supporting cell culture of primary tissues. An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also be employed for inducing tissue of mesodermal origin to differentiate in early embryos.

[0878] An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also increase or decrease the differentiation or proliferation of embryonic stem cells, besides, as discussed above, hematopoietic lineage.

[0879] An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also be used to modulate mammalian characteristics, such as body height, weight, hair color, eye color, skin, percentage of adipose tissue, pigmentation, size, and shape (e.g., cosmetic surgery). Similarly, an albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may be used to modulate mammalian metabolism affecting catabolism, anabolism, processing, utilization, and storage of energy.

[0880] An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may be used to change a mammal's mental state or physical state by influencing biorhythms, caricadic rhythms, depression (including depressive disorders), tendency for violence, tolerance for pain, reproductive capabilities (preferably by Activin or Inhibin-like activity), hormonal or endocrine levels, appetite, libido, memory, stress, or other cognitive qualities.

[0881] An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also be used as a food additive or preservative, such as to increase or decrease storage capabilities, fat content, lipid, protein, carbohydrate, vitamins, minerals, cofactors or other nutritional components.

[0882] The above-recited applications have uses in a wide variety of hosts. Such hosts include, but are not limited to, human, murine, rabbit, goat, guinea pig, camel, horse, mouse, rat, hamster, pig, micro-pig, chicken, goat, cow, sheep, dog, cat, non-human primate, and human. In specific embodiments, the host is a mouse, rabbit, goat, guinea pig, chicken, rat, hamster, pig, sheep, dog or cat. In preferred embodiments, the host is a mammal. In

most preferred embodiments, the host is a human.

[0883] Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

[0884] Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the alterations detected in the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

EXAMPLE 1: Generation of pScNHSA and pScCHSA.

[0885] The vectors pScNHSA (ATCC Deposit No. PTA-3279) and pScCHSA (ATCC Deposit No. PTA-3276) are derivatives of pPPC0005 (ATCC Deposit No. PTA-3278) and are used as cloning vectors into which polynucleotides encoding a therapeutic protein or fragment or variant thereof is inserted adjacent to and in translation frame with polynucleotides encoding human serum albumin "HSA". pScCHSA may be used for generating Therapeutic protein-HSA fusions, while pScNHSA may be used to generate HSA-Therapeutic protein fusions.

Generation of pScCHSA: albumin fusion with the albumin moiety C-terminal to the therapeutic portion.

[0886] A vector to facilitate cloning DNA encoding a Therapeutic protein N-terminal to DNA encoding the mature albumin protein was made by altering the nucleic acid sequence that encodes the chimeric HSA signal peptide in pPPC0005 to include the *Xho* I and *Cla* I restriction sites.

[0887] First, the *Xho* I and *Cla* I sites inherent to pPPC0005 (located 3' of the ADH1 terminator sequence) were eliminated by digesting pPPC0005 with *Xho* I and *Cla* I, filling in the sticky ends with T4 DNA polymerase, and religating the blunt ends to create pPPC0006.

[0888] Second, the *Xho* I and *Cla* I restriction sites were engineered into the nucleic acid sequence that encodes the signal peptide of HSA (a chimera of the HSA leader and a

kex2 site from mating factor alpha, "MAF") in pPPC0006 using two rounds of PCR. In the first round of PCR, amplification with primers shown as SEQ ID NO:1039 and SEQ ID NO:1040 was performed. The primer whose sequence is shown as SEQ ID NO:1039 comprises a nucleic acid sequence that encodes part of the signal peptide sequence of HSA, a kex2 site from the mating factor alpha leader sequence, and part of the amino-terminus of the mature form of HSA. Four point mutations were introduced in the sequence, creating the *Xho* I and *Cla* I sites found at the junction of the chimeric signal peptide and the mature form of HSA. These four mutations are underlined in the sequence shown below. In pPPC0005 the nucleotides at these four positions from 5' to 3' are T, G, T, and G.

5'-GCCTCGAGAAAAGAGATGCACACAAGAGTGAGGTTGCTCATCGATTTAAAGAT TTGGG-3' (SEQ ID NO:1039) and

5'-AATCGATGAGCAACCTCACTCTTGTGTGCATCTCTTTTCTCGAGGCTCCTGGAA TAAGC-3' (SEQ ID NO:1040). A second round of PCR was then performed with an upstream flanking primer, 5'-TACAAACTTAAGAGTCCAATTAGC-3' (SEQ ID NO:1041) and a downstream flanking primer

5'-CACTTCTCTAGAGTGGTTTCATATGTCTT-3' (SEQ ID NO:1042). The resulting PCR product was then purified and digested with *Afl* II and *Xba* I and ligated into the same sites in pPPC0006 creating pScCHSA. The resulting plasmid has *Xho* I and *Cla* I sites engineered into the signal sequence. The presence of the *Xho* I site creates a single amino acid change in the end of the signal sequence from LDKR to LEKR. The D to E change will not be present in the final albumin fusion protein expression plasmid when a nucleic acid sequence comprising a polynucleotide encoding the Therapeutic portion of the albumin fusion protein with a 5' *Sal* I site (which is compatible with the *Xho* I site) and a 3' *Cla* I site is ligated into the *Xho* I and *Cla* I sites of pScCHSA. Ligation of *Sal* I to *Xho* I restores the original amino acid sequence of the signal peptide sequence. DNA encoding the Therapeutic portion of the albumin fusion protein may be inserted after the Kex2 site (Kex2 cleaves after the dibasic amino acid sequence KR at the end of the signal peptide) and prior to the *Cla* I site.

Generation of pScNHSA: albumin fusion with the albumin moiety N-terminal to the therapeutic portion.

[0889] A vector to facilitate cloning DNA encoding a Therapeutic protein portion C-terminal to DNA encoding the mature albumin protein, was made by adding three, eight-

base-pair restriction sites to pScCHSA. The *Asc* I, *Fse* I, and *Pme* I restriction sites were added in between the *Bsu36* I and *Hind* III sites at the end of the nucleic acid sequence encoding the mature HSA protein. This was accomplished through the use of two complementary synthetic primers containing the *Asc* I, *Fse* I, and *Pme* I restriction sites underlined (SEQ ID NO:1043 and SEQ ID NO:1044). 5'-AAGCTGCCTTAGGCTTATAATAAGGCGCGCCGGCCGGCCGTTTAAACTAAGCT TAATTCT-3' (SEQ ID NO:1043) and 5-AGAATTAAGCTTAGTTTAAACGGCCGGCCGGCGCGCCTTATTATAAGCCTAAG GCAGCTT-3' (SEQ ID NO:1044). These primers were annealed and digested with *Bsu36* I and *Hind* III and ligated into the same sites in pScCHSA creating pScNHSA.

EXAMPLE 2: General Construct Generation for Yeast Transformation.

[0890] The vectors pScNHSA and pScCHSA may be used as cloning vectors into which polynucleotides encoding a therapeutic protein or fragment or variant thereof is inserted adjacent to polynucleotides encoding mature human serum albumin "HSA". pScCHSA is used for generating Therapeutic protein-HSA fusions, while pScNHSA may be used to generate HSA-Therapeutic protein fusions.

Generation of albumin fusion constructs comprising HSA-Therapeutic protein fusion products.

[0891] DNA encoding a Therapeutic protein (e.g., sequences shown in SEQ ID NO:X or known in the art) may be PCR amplified using the primers which facilitate the generation of a fusion construct (e.g., by adding restriction sites, encoding seamless fusions, encoding linker sequences, etc.) For example, one skilled in the art could design a 5' primer that adds polynucleotides encoding the last four amino acids of the mature form of HSA (and containing the *Bsu*36I site) onto the 5' end of DNA encoding a Therapeutic protein; and a 3' primer that adds a STOP codon and appropriate cloning sites onto the 3' end of the Therapeutic protein coding sequence. For instance, the forward primer used to amplify DNA encoding a Therapeutic protein might have the sequence, 5'-aagctGCCTTAGGCTTA$(N)_{15}$-3' (SEQ ID NO:1045) where the underlined sequence is a *Bsu*36I site, the upper case nucleotides encode the last four amino acids of the mature HSA protein (ALGL), and $(N)_{15}$ is identical to the first 15 nucleotides encoding the Therapetic protein of interest. Similarly, the reverse primer used to amplify DNA encoding a Therapeutic protein might have the

sequence, 5'-GCGCGC*GTTTAAAC*GGCCGGCCGGCGCGCCTTATTA$(N)_{15}$-3' (SEQ ID NO:1046) where the italicized sequence is a *Pme* I site, the double underlined sequence is an *Fse* I site, the singly underlined sequence is an *Asc* I site, the boxed nucleotides are the reverse complement of two tandem stop codons, and $(N)_{15}$ is identical to the reverse complement of the last 15 nucleotides encoding the Therapeutic protein of interest. Once the PCR product is amplified it may be cut with *Bsu*36I and one of (*Asc* I, *Fse* I, or *Pme* I) and ligated into pScNHSA.

[0892] The presence of the *Xho* I site in the HSA chimeric leader sequence creates a single amino acid change in the end of the chimeric signal sequence, i.e. the HSA-kex2 signal sequence, from LDKR (SEQ ID NO:2139) to LEKR (SEQ ID NO:2140).

Generation of albumin fusion constructs comprising gene-HSA fusion products.

[0893] Similar to the method described above, DNA encoding a Therapeutic protein may be PCR amplified using the following primers: A 5' primer that adds polynucleotides containing a *Sal*I site and encoding the last three amino acids of the HSA leader sequence, DKR, onto the 5' end of DNA encoding a Therapeutic protein; and a 3' primer that adds polynucleotides encoding the first few amino acids of the mature HSA containing a *Cla* I site onto the 3' end of DNA encoding a Therapeutic protein. For instance, the forward primer used to amplify the DNA encoding a Therapeutic protein might have the sequence, 5'-aggagcgtcGACAAAAGA$(N)_{15}$-3' (SEQ ID NO:1047) where the underlined sequence is a *Sal* I site, the upper case nucleotides encode the last three amino acids of the HSA leader sequence (DKR), and $(N)_{15}$ is identical to the first 15 nucleotides encoding the Therapetic protein of interest. Similarly, the reverse primer used to amplify the DNA encoding a Therapeutic protein might have the sequence, 5'-CTTTAA*ATCGAT*GAGCAACCTCACTCTTGTGTGCATC$(N)_{15}$-3'(SEQ ID NO:1048) where the italicized sequence is a *Cla* I site, the underlined nucleotides are the reverse complement of the DNA encoding the first 9 amino acids of the mature form of HSA (DAHKSEVAH, SEQ ID NO:1106), and $(N)_{15}$ is identical to the reverse complement of the last 15 nucleotides encoding the Therapeutic protein of interest. Once the PCR product is amplified it may be cut with *Sal* I and *Cla* I and ligated into pScCHSA digested with *Xho* I and *Cla* I. A different signal or leader sequence may be desired, for example, invertase "INV" (Swiss-Prot Accession P00724), mating factor alpha "MAF" (Genbank Accession AAA18405), MPIF (Geneseq AAF82936), Fibulin B (Swiss-Prot Accession P23142),

Clusterin (Swiss-Prot Accession P10909), Insulin-Like Growth Factor- Binding Protein 4 (Swiss-Prot Accession P22692), and permutations of the HSA leader sequence can be subcloned into the appropriate vector by means of standard methods known in the art.

Generation of albumin fusion construct compatible for expression in yeast S. cerevisiae.

[0894] The *Not* I fragment containing the DNA encoding either an N-terminal or C-terminal albumin fusion protein generated from pScNHSA or pScCHSA may then be cloned into the *Not* I site of pSAC35 which has a LEU2 selectable marker. The resulting vector is then used in transformation of a yeast *S. cerevisiae* expression system.

EXAMPLE 3: General Expression in Yeast *S. cerevisiae*.

[0895] An expression vector compatible with yeast expression can be transformed into yeast *S. cerevisiae* by lithium acetate transformation, electroporation, or other methods known in the art and or as described in part in Sambrook, Fritsch, and Maniatis. 1989. "Molecular Cloning: A Laboratory Manual, 2nd edition", volumes 1-3, and in Ausubel et al. 2000. Massachusetts General Hospital and Harvard Medical School "Current Protocols in Molecular Biology", volumes 1-4. The expression vectors are introduced into *S. cerevisiae* strains DXY1, D88, or BXP10 by transformation, individual transformants can be grown, for example, for 3 days at 30°C in 10 mL YEPD (1% w/v yeast extract, 2 % w/v, peptone, 2 % w/v, dextrose), and cells can be collected at stationary phase after 60 hours of growth. Supernatants are collected by clarifying cells at 3000g for 10 minutes.

[0896] pSAC35 (Sleep et al., 1990, Biotechnology 8:42 and see Figure 3) comprises, in addition to the LEU2 selectable marker, the entire yeast 2 μm plasmid to provide replication functions, the PRB1 promoter, and the ADH1 termination signal.

EXAMPLE 4: General Purification of an Albumin Fusion Protein Expressed from an Albumin Fusion in Yeast *S. cerevisiae*.

[0897] In preferred embodiments, albumin fusion proteins of the invention comprise the mature form of HSA fused to either the N- or C- terminus of the mature form of a therapeutic protein or portions thereof (e.g., the mature form of a therapeutic protein listed in Table 1, or the mature form of a therapeutic protein shown in Table 2 as SEQ ID NO:Z). In one embodiment of the invention, albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the

host used for expression. In a preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature albumin fusion protein is secreted directly into the culture medium. Albumin fusion proteins of the invention preferably comprise heterologous signal sequences (e.g., the non-native signal sequence of a particular therapeutic protein) including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. Especially preferred as those signal sequence listed in Table 2 and/or the signal sequence listed in the "Expression of Fusion Proteins" and/or "Additional Methods of Recombinant and Synthetic Production of Albumin Fusion Proteins" section of the specification, above. In preferred embodiments, the fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

[0898] Albumin fusion proteins expressed in yeast as described above can be purified on a small-scale over a Dyax peptide affinity column as follows. Supernatants from yeast expressing an albumin fusion protein is diafiltrated against 3 mM phosphate buffer pH 6.2, 20 mM NaCl and 0.01% Tween 20 to reduce the volume and to remove the pigments. The solution is then filtered through a 0.22 μm device. The filtrate is loaded onto a Dyax peptide affinity column. The column is eluted with 100 mM Tris/HCl, pH 8.2 buffer. The peak fractions containing protein are collected and analyzed on SDS-PAGE after concentrating 5-fold.

[0899] For large scale purification, the following method can be utilized. The supernatant in excess of 2 L is diafiltered and concentrated to 500 mL in 20 mM Tris/HCl pH 8.0. The concentrated protein solution is loaded onto a pre-equilibrated 50 mL DEAE-Sepharose Fast Flow column, the column is washed, and the protein is eluted with a linear gradient of NaCl from 0 to 0.4 M NaCl in 20 mM Tris/HCl, pH 8.0. Those fractions containing the protein are pooled, adjusted to pH 6.8 with 0.5 M sodium phosphate (NaH_2PO_4). A final concentration of 0.9 M $(NH_4)_2SO_4$ is added to the protein solution and the whole solution is loaded onto a pre-equilibrated 50 mL Butyl650S column. The protein is eluted with a linear gradient of ammonium sulfate (0.9 to 0 M $(NH_4)_2SO_4$). Those fractions with the albumin fusion are again pooled, diafiltered against 10 mM Na_2HPO_4/citric acid buffer pH 5.75, and loaded onto a 50 mL pre-equilibrated SP-Sepharose Fast Flow column. The protein is eluted with a NaCl linear gradient from 0 to 0.5 M. The fractions containing

the protein of interest are combined, the buffer is changed to 10 mM Na_2HPO_4/citric acid pH 6.25 with an Amicon concentrator, the conductivity is < 2.5 mS/cm. This protein solution is loaded onto a 15 mL pre-equilibrated Q-Sepharose high performance column, the column is washed, and the protein is eluted with a NaCl linear gradient from 0 to 0.15 M NaCl. The purified protein can then be formulated into a specific buffer composition by buffer exchange.

EXAMPLE 5: General Construct Generation for Mammalian Cell Transfection.

Generation of albumin fusion construct compatible for expression in mammalian cell-lines.

[0900] Albumin fusion constructs can be generated in expression vectors for use in mammalian cell culture systems. DNA encoding a therapeutic protein can be cloned N-terminus or C-terminus to HSA in a mammalian expression vector by standard methods known in the art (e.g., PCR amplification, restriction digestion, and ligation). Once the expression vector has been constructed, transfection into a mammalian expression system can proceed. Suitable vectors are known in the art including, but not limited to, for example, the pC4 vector, and/or vectors available from Lonza Biologics, Inc. (Portsmouth, NH).

[0901] The DNA encoding human serum albumin has been cloned into the pC4 vector which is suitable for mammalian culture systems, creating plasmid pC4:HSA (ATCC Deposit # PTA-3277). This vector has a DiHydroFolate Reductase, "DHFR", gene that will allow for selection in the presence of methotrexate.

[0902] The pC4:HSA vector is suitable for expression of albumin fusion proteins in CHO cells. For expression, in other mammalian cell culture systems, it may be desirable to subclone a fragment comprising, or alternatively consisting of, DNA which encodes for an albumin fusion protein into an alternative expression vector. For example, a fragment comprising, or alternatively consisting, of DNA which encodes for a mature albumin fusion protein may be subcloned into another expression vector including, but not limited to, any of the mammalian expression vectors described herein.

[0903] In a preferred embodiment, DNA encoding an albumin fusion construct is subcloned into vectors provided by Lonza Biologics, Inc. (Portsmouth, NH) by procedures known in the art for expression in NS0 cells.

Generation of albumin fusion constructs comprising HSA-Therapeutic Protein fusion products.

[0904] Using pC4:HSA (ATCC Deposit # PTA-3277), albumin fusion constructs can

be generated in which the Therapeutic protein portion is C terminal to the mature albumin sequence. For example, one can clone DNA encoding a Therapeutic protein of fragment or variant thereof between the *Bsu* 36I and *Asc* I restriction sites of the vector. When cloning into the *Bsu* 36I and *Asc* I, the same primer design used to clone into the yeast vector system (SEQ ID NO:1045 and 1046) may be employed (see Example 2).

Generation of albumin fusion constructs comprising gene-HSA fusion products.

[0905] Using pC4:HSA (ATCC Deposit # PTA-3277), albumin fusion constructs can be generated in which a Therapeutic protein portion is cloned N terminal to the mature albumin sequence. For example, one can clone DNA encoding a Therapeutic protein that has its own signal sequence between the *Bam* HI (or *Hind* III) and *Cla* I sites of pC4:HSA. When cloning into either the *Bam* HI or *Hind* III site, it is preferrable to include a Kozak sequence (CCGCCACCATG, SEQ ID NO:1107) prior to the translational start codon of the DNA encoding the Therapeutic protein. If a Therapeutic protein does not have a signal sequence, DNA encoding that Therapeutic protein may be cloned in between the *Xho* I and *Cla* I sites of pC4:HSA. When using the *Xho* I site, the following 5' (SEQ ID NO:1052) and 3' (SEQ ID NO:1053) exemplary PCR primers may be used:

5'-CCGCCGCTCGAGGGGTGTGTTTCGTCGA$(N)_{18}$-3' (SEQ ID NO: 1052)

5'-AGTCCCATCGATGAGCAACCTCACTCTTGTGTGCATC$(N)_{18}$-3' (SEQ ID NO:1053)

[0906] In the 5' primer (SEQ ID NO:1052), the underlined sequence is a *Xho* I site; and the *Xho* I site and the DNA following the *Xho* I site code for the last seven amino acids of the leader sequence of natural human serum albumin. In SEQ ID NO:1052, "$(N)_{18}$" is DNA identical to the first 18 nucleotides encoding the Therapeutic protein of interest. In the 3' primer (SEQ ID NO:1053), the underlined sequence is a *Cla* I site; and the *Cla* I site and the DNA following it are the reverse complement of the DNA encoding the first 10 amino acids of the mature HSA protein (SEQ ID NO:1038). In SEQ ID NO:1053 "$(N)_{18}$" is the reverse complement of DNA encoding the last 18 nucleotides encoding the Therapeutic protein of interest. Using these two primers, one may PCR amplify the Therapeutic protein of interest, purify the PCR product, digest it with *Xho* I and *Cla* I restriction enzymes and clone it into the *Xho* I and *Cla* I sites in the pC4:HSA vector.

[0907] If an alternative leader sequence is desired, the native albumin leader sequence can be replaced with the chimeric albumin leader, i.e., the HSA-kex2 signal peptide, or an alternative leader by standard methods known in the art. (For example, one skilled in the art

could routinely PCR amplify an alternate leader and subclone the PCR product into an albumin fusion construct in place of the albumin leader while maintaining the reading frame)

.

EXAMPLE 6: General Expression in Mammalian Cell-Lines.

[0908] An albumin fusion construct generated in an expression vector compatible with expression in mammalian cell-lines can be transfected into appropriate cell-lines by calcium phosphate precipitation, lipofectamine, electroporation, or other transfection methods known in the art and/or as described in Sambrook, Fritsch, and Maniatis. 1989. "Molecular Cloning: A Laboratory Manual, 2nd edition" and in Ausubel et al. 2000. Massachusetts General Hospital and Harvard Medical School "Current Protocols in Molecular Biology", volumes 1-4. The transfected cells are then selected for by the presence of a selecting agent determined by the selectable marker in the expression vector.

[0909] The pC4 expression vector (ATCC Accession No. 209646) is a derivative of the plasmid pSV2-DHFR (ATCC Accession No. 37146). pC4 contains the strong promoter Long Terminal Repeats "LTR" of the Rous Sarcoma Virus (Cullen et al., March 1985, Molecular and Cellular Biology, 438-447) and a fragment of the CytoMegaloVirus "CMV"-enhancer (Boshart et al., 1985, Cell 41: 521-530). The vector also contains the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene, and the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary "CHO" cells or other cell-lines lacking an active DHFR gene are used for transfection. Transfection of an albumin fusion construct in pC4 into CHO cells by methods known in the art will allow for the expression of the albumin fusion protein in CHO cells, followed by leader sequence cleavage, and secretion into the supernatant. The albumin fusion protein is then further purified from the supernatant.

[0910] The pEE12.1 expression vector is provided by Lonza Biologics, Inc. (Portsmouth, NH) and is a derivative of pEE6 (Stephens and Cockett, 1989, Nucl. Acids Res. 17: 7110). This vector comprises a promoter, enhancer and complete 5'-untranslated region of the Major Immediate Early gene of the human CytoMegaloVirus, "hCMV-MIE" (International Publication # WO89/01036), upstream of a sequence of interest, and a Glutamine Synthetase gene (Murphy et al., 1991, Biochem J. 227: 277-279; Bebbington et al., 1992, Bio/Technology 10:169-175; US patent US 5,122,464) for purposes of selection of transfected cells in selective methionine sulphoximine containing medium. Transfection of

albumin fusion constructs made in pEE12.1 into NS0 cells (International Publication # WO86/05807) by methods known in the art will allow for the expression of the albumin fusion protein in NS0 cells, followed by leader sequence cleavage, and secretion into the supernatant. The albumin fusion protein is then further purified from the supernatant using techniques described herein or otherwise known in the art.

[0911] Expression of an albumin fusion protein may be analyzed, for example, by SDS-PAGE and Western blot, reversed phase HPLC analysis, or other methods known in the art.

[0912] Stable CHO and NS0 cell-lines transfected with albumin fusion constructs are generated by methods known in the art (e.g., lipofectamine transfection) and selected, for example, with 100 nM methotrexate for vectors having the DiHydroFolate Reductase 'DHFR' gene as a selectable marker or through growth in the absence of glutamine. Expression levels can be examined for example, by immunoblotting, primarily, with an anti-HSA serum as the primary antibody, or, secondarily, with serum containing antibodies directed to the Therapeutic protein portion of a given albumin fusion protein as the primary antibody.

[0913] Expression levels are examined by immunoblot detection with anti-HSA serum as the primary antibody. The specific productivity rates are determined via ELISA in which the capture antibody can be a monoclonal antibody towards the therapeutic protein portion of the albumin fusion and the detecting antibody can be the monoclonal anti-HSA-biotinylated antibody *(or vice versa),* followed by horseradish peroxidase/streptavidin binding and analysis according to the manufacturer's protocol.

EXAMPLE 7: General Purification of an Albumin Fusion Protein Expressed from an Albumin Fusion Construct in Mammalian Cell-lines.

[0914] In preferred embodiments, albumin fusion proteins of the invention comprise the mature form of HSA fused to either the N- or C- terminus of the mature form of a therapeutic protein or portions thereof (e.g., the mature form of a therapeutic protein listed in Table 1, or the mature form of a therapeutic protein shown in Table 2 as SEQ ID NO:Z). In one embodiment of the invention, albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature albumin fusion protein is secreted directly into the

culture medium. Albumin fusion proteins of the invention preferably comprise heterologous signal sequences (e.g., the non-native signal sequence of a particular therapeutic protein) including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. Especially preferred as those signal sequence listed in Table 2 and/or the signal sequence listed in the "Expression of Fusion Proteins" and/or "Additional Methods of Recombinant and Synthetic Production of Albumin Fusion Proteins" section of the specification, above. In preferred embodiments, the fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

[0915] Albumin fusion proteins from mammalian cell-line supernatants are purified according to different protocols depending on the expression system used.

Purification from CHO and 293T cell-lines.

[0916] Purification of an albumin fusion protein from CHO cell supernatant or from transiently transfected 293T cell supernatant may involve initial capture with an anionic HQ resin using a sodium phosphate buffer and a phosphate gradient elution, followed by affinity chromatography on a Blue Sepharose FF column using a salt gradient elution. Blue Sepharose FF removes the main BSA/fetuin contaminants. Further purification over the Poros PI 50 resin with a phosphate gradient may remove and lower endotoxin contamination as well as concentrate the albumin fusion protein.

Purification from NS0 cell-line.

[0917] Purification of an albumin-fusion protein from NS0 cell supernatant may involve Q-Sepharose anion exchange chromatography, followed by SP-sepharose purification with a step elution, followed by Phenyl-650M purification with a step elution, and, ultimately, diafiltration.

[0918] The purified protein may then be formulated by buffer exchange.

EXAMPLE 8: Construct ID 1966, EPO-HSA, Generation.

[0919] Construct ID 1966, pC4.EPO:M1-D192.HSA, encodes for an EPO-HSA fusion protein which comprises the EPO native leader sequence as well as the mature EPO

protein with the exception of the final Arg residue, i.e., M1-D192, fused to the amino-terminus of the mature form of HSA cloned into the mammalian expression vector pC4.

Cloning of EPO cDNA for construct 1966

[0920] The DNA encoding EPO was amplified with primers EPO1 and EPO2, described below, cut with *Bam* HI/*Cla* I, and ligated into *Bam* HI/*Cla* I cut pC4:HSA. Construct ID #1966 encodes an albumin fusion protein containing the leader sequence and the mature form of EPO, followed by the mature HSA protein (see SEQ ID NO:297 for construct 1966 in table 2).

[0921] Two oligonucleotides suitable for PCR amplification of the polynucleotide encoding the full length EPO including the natural leader sequence (SEQ ID NO:81, table 2), EPO1 and EPO2, were synthesized.

EPO1: 5'-GACT*GGATCCGCCACCATG***GGGGTGCACGAATGTCCTGCCTGGCTGTGGCTTCT CCTGTCCCTGCTGTCGCTCCCTCTGGGCCTCCCAGTCCTGGGCGCCCCACCACGCCTCATCT GTGAC**-3' (SEQ ID NO: 1122)

EPO2: 5'- AGTCCCATCGATGAGCAACCTCACTCTTGTGTGCATC**GTCCCCTGTCCTGCAGGC CTCC**–3' (SEQ ID NO: 804)

[0922] EPO1 incorporates a *Bam* HI cloning site (shown in italics) and attaches a kozak sequence (shown double underlined) prior to the DNA encoding the first 35 amino acids of the ORF of the full-length EPO. In EPO2, the underlined sequence is a *Cla* I site; and the *Cla* I site and the DNA following it are the reverse complement of DNA encoding the first 10 amino acids of the mature HSA protein (SEQ ID NO:1038). In EPO2, the bolded sequence is the reverse complement of the last 22 nucleotides encoding amino acid residues Glu-186 to Asp-192 of the full-length form of EPO, with the exception of the final Arg residue. Using these two primers, the full-length EPO protein, with the exception of the final Arg residue, was PCR amplified. Annealing and extension temperatures and times must be empirically determined for each specific primer pair and template.

[0923] The PCR product was purified (for example, using Wizard PCR Preps DNA Purification System (Promega Corp)) and then digested with *Bam* HI and *Cla* I. After further purification of the *Bam* HI-*Cla* I fragment by gel electrophoresis, the product was cloned into *Bam* HI/*Cla* I digested pC4:HSA to produce construct ID # 1966.

[0924] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing confirmed the presence of the expected EPO sequence (see below).

[0925] EPO albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of EPO lacking the final Arg residue, i.e., Ala-28 to Asp-192. In one embodiment of the invention, EPO albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature EPO albumin fusion protein is secreted directly into the culture medium. EPO albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, EPO albumin fusion proteins of the invention comprise the native EPO signal sequence. In further preferred embodiments, the EPO albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 1966.

Expression in either 293T or CHO cells.

[0926] Construct 1966 was transfected into either 293T cells or CHO cells by methods known in the art (e.g., lipofectamine transfection) and selected with 100 nM methotrexate (see Example 6). Expression levels were examined by immunoblot detection with anti-HSA serum as the primary antibody, and the specific productivity rates were determined via ELISA using a monoclonal anti-human EPO antibody (Research Diagnostics, Inc.) for capture and a Biotrend monoclonal anti-HSA-biotinylated antibody for detection, followed by horseradish peroxidase/streptavidin binding and analysis.

Purification from 293T cell supernatant.

[0927] The 293T cell supernatant containing the secreted EPO-HSA fusion protein expressed from construct ID #1966 in 293T cells was purified as described in Example 7. Specifically, initial capture was performed with an anionic HQ-50 resin at pH 7.2 using a step elution, followed by Blue sepharose FF chromatography again employing a step elution at pH 7.2. The pooled fractions were passed over the HQ-50 resin again using a step elution. The eluted sample was then loaded onto the Phenyl-650M column and eluted with a gradient

elution at pH 7.2. The eluted sample was passed over the HQ-50 resin for a third time. The fractions of interest were diafiltrated into 50 mM Na_2HPO_4 + 200 mM NaCl pH 7.2. N-terminal sequencing generated the amino-terminus sequence (i.e., APPRLI) of the mature form of EPO. A protein of approximate MW of 90 kDa was obtained. A final yield of 0.42 mg protein per litre of 293T cell supernatant was obtained.

Purification from CHO cell supernatant.

[0928] The cell supernatant containing the EPO-albumin fusion protein expressed from construct ID #1966 in CHO cells was purified as described in Example 7. Specifically, initial capture of a concentrated 1.4 L sample was performed with an anionic Poros HQ 50 resin using a sodium phosphate buffer and a phosphate gradient elution (0 – 100 mM sodium phosphate, pH 7.2). Prior to loading the column, the sample was diluted with 3 mM phosphate until the conductivity was lower than 5.0 mS, as was the case for further column chromatography purifications. The HQ resin was equilibrated with 10 mM sodium phosphate, pH 7.2 prior to sample loading. EPO-HSA eluted at 20 mS, or 50 mM sodium phosphate. The second purification step involved affinity chromatography. The combined fractions from the previous HQ resin elution, adjusted for a conductivity <5 mS using 3 mM phosphate pH 7.2 buffer, were loaded onto a Blue Sepharose FF column equilibrated with 125 mM NaCl, 15 mM sodium phosphate, pH 7.2. A salt gradient of 0 – 3 M NaCl eluted EPO-HSA between 0.5 M and 1.0 M NaCl. Blue Sepharose FF removes the main BSA/fetuin contaminants. The conductivity of the desired fractions was again adjusted for, and the pooled fractions were loaded onto a third column containing Poros PI 50 resin which removes and lowers endotoxin contamination as well as concentrates the EPO-HSA protein. The resin was equilibrated with 25 mM NaCl, 10 mM sodium phosphate, pH 7.2. EPO-HSA was eluted with a 10 mM – 100 mM phosphate gradient. The final buffer composition was 100 mM NaCl, 20 mM Na_2HPO_4, pH 7.2. An approximate protein MW of 87.7 kDa was obtained. A final yield of 8.9 mg protein per liter of supernatant was obtained. N-Terminal sequencing generated the sequence APPRL which corresponds to the amino-terminus of the mature form of EPO.

In vitro TF-1 cell proliferation assay.

Method

[0929] The biological activity of an EPO albumin fusion protein can be measured in an *in vitro* TF-1 cell proliferation assay. The TF-1 cell-line was established by Kitamura et

al. (Kitamura, T. et al., 1989, J. Cell. Physiol., 140: 323 – 334). The TF-1 cells were derived from a heparinized bone marrow aspiration sample from a 35 year old Japanese male with severe pancytopenia. The TF-1 cell-line provides a good system for investigating the proliferation and differentiation of myeloid progenitor cells as a result of its responsiveness to multiple cytokines.

[0930] TF-1 cell proliferation assay (Kitamura, T. et al., 1989, J. Cell. Physiol., 140: 323 – 334): Human TF-1 cells (ATCC # CRL-2003) are expanded in RPMI 1640 media containing 10% FBS, 1X pen-strep, 1X L-glutamine, and 2 ng/mL human GM-CSF to a maximum density of 1 x 10^6 cells/mL. Cells are passaged every 2-3 days by diluting 1:10 or 1:20 in fresh medium. On the day of the assay initiation, cells are washed in a 50 mL volume of RPMI 1640/10% FBS three times to remove GM-CSF and are resuspended at $1x10^5$ cells/mL in RPMI 1640/10% FBS. Cells are plated at 10,000 cells/well in flat-bottom TC-treated 96-well plates. Three-fold serial dilutions of control protein are made in RPMI 1640/10% FBS in a range of 10 U/mL to 0.001 U/mL (final concentration) and three-fold serial dilutions of an albumin fusion protein are made in RPMI 1640/10% FBS in a range of 100 ng/mL to 0.01 ng/mL (final concentration) where 1 U = 10 ng protein; 0.1 mL of each dilution is added to triplicate wells containing cells for a final volume of 0.2 mL in each well. Cell proliferation response to the control protein and the albumin fusion protein is determined by measuring incorporation of ^{3}H-thymidine (0.5 uCi/well). The assay is carried out at incubation times of 24, 48, or 72 hours prior to and for 4 – 24 hours after the addition of ^{3}H-thymidine. Since only a portion of the molar weight of an albumin fusion protein is actually a therapeutic protein molecule (i.e., the therapeutic protein portion of the fusion), in some cases dilutions may also be adjusted for the molar ratio.

In vitro TF-1 cell proliferation assay for the albumin fusion protein encoded by construct 1966.

Method

[0931] TF-1 cell proliferation assay: Human TF-1 cells (ATCC # CRL-2003) were expanded in RPMI 1640 media containing 10% FBS, 1X pen-strep, 1X L-glutamine, and 2 ng/mL human GM-CSF to a maximum density of 1 x 10^6 cells/mL. Cells were passaged every 2-3 days by diluting 1:10 or 1:20 in fresh medium. On the day of the assay initiation, cells were washed in a 50 mL volume of RPMI 1640/10% FBS three times to remove GM-CSF and were resuspended at $1x10^5$ cells/mL in RPMI 1640/10% FBS. Cells were plated at

10,000 cells/well in flat-bottom TC-treated 96-well plates. Three-fold serial dilutions of hrEPO (R&D Systems; Research Diagnostics Inc., RDI) were made in RPMI 1640/10% FBS in a range of 10 U/mL to 0.001 U/mL (final concentration) and three-fold serial dilutions of the EPO albumin fusion protein were made in RPMI 1640/10% FBS in a range of 100 ng/mL to 0.01 ng/mL (final concentration) where 1 U = 10 ng protein; 0.1 mL of each dilution was added to triplicate wells containing cells for a final volume of 0.2 mL in each well. Cell proliferation response to hrEPO and EPO albumin protein was determined by measuring incorporation of ^{3}H-thymidine (0.5 μCi/well). The assay was carried out at incubation times of 24, 48, or 72 hours prior to and for 4 – 24 hours after the addition of ^{3}H-thymidine. Since only 1/3 of the molar weight of the EPO albumin fusion protein is actually an EPO molecule, in some cases dilutions made were also to adjust for the molar ratio.

Results

[0932] Supernatants from 293T cells expressing construct 1966 or >90% purified EPO-HSA albumin fusion protein derived from CHO cells expressing construct 1966 were tested in the above assay for EPO activity. On average, an EC50 of greater than 5 fold of that of rhEPO was established (see Figure 4).

In vivo Harlan mouse model for measuring hematocrit.

Methods

[0933] This mouse model provides the means to measure the therapeutic activity of a protein *in vivo* by measuring its effect on the hematocrit.

[0934] An *in vivo* mouse model, i.e., 6 – 8 week old female DBA/2NHsd mice (Harlan), has been established to monitor the effect on hematocrit upon administration of a control protein at 2 μg/kg and at other concentrations or an albumin fusion protein at 30 μg/kg and at other concentrations daily or every other day for 7 days either intravenously, intraperitoneally, or subcutaneously. Hematocrit is determined by sticking the tail vein with a needle, collecting the blood with a heparinized microcapillary tube, and then spinning the tubes throughout the experimental time-frame. Also, for certain experiments, the spleen is harvested and weighed. Other dosing schedules are known within the art and can readily be adapted for use in this assay.

The activity of the albumin fusion protein encoded by construct 1966 can be assayed using an in vivo Harlan mouse model for measuring hematocrit.

Methods

[0935] An *in vivo* mouse model of 6 – 8 week old female DBA/2NHsd mice (Harlan) was used to monitor the extent of EPO activity upon administration of rhEPO (Research Diagnostics, Inc., cat # RDI-PB11965) at doses of 0.5, 1.5, 4.5, and 12 μg/kg on days 0, 2, 4 and 6 and upon administration of the purified EPO albumin fusion protein encoded by construct 1966 at concentrations of 2, 6, 18, and 54 μg/kg on days 0, 2, 4, and 6 subcutaneously, "SC". Hematocrit was determined by sticking the tail vein with a needle on days 0 and 7, collecting the blood with a heparinized microcapillary tube, and then spinning the tubes throughout the experimental time-frame. The higher doses of the EPO albumin fusion protein is a rough equimolar comparison with the control recombinant human EPO, "rhEPO" (Research Diagnostics, Inc., cat # RDI-PB11965).

Results

[0936] There was a significant increase in hematocrit (see Figure 5) from day 0 to day 7 for animals treated with either recombinant human EPO or EPO albumin fusion proteins. However, the EPO albumin fusion protein encoded by construct 1966 appeared to have a more drastic effect on hematocrit levels than the rhEPO control. Subcutaneous administration of 3 doses/week of 52 μg/kg, or 1 dose/week of 156 μg/kg, of the EPO albumin fusion protein encoded by construct 1966 caused a greater than or equal to 40% change in hematocrit from day 0 to day 8 (see Figure 6). The % change in hematocrit was either maintained close to 40% for the triple dose or subdued to ~20% for the single dose on day 14 as opposed to a decline from close to 30% to <10% for a 3 dose subcutaneous administration of 12 μg/kg of rhEPO in a week. The elevated hematocrit appears to be maintained with the EPO albumin fusion protein encoded by construct 1966 over a period of a week after the last subcutaneous administration in comparison with the hematocrit levels induced by the rhEPO protein which declines back to more normal levels.

[0937] DBA mice injected intravenously with a 150 μg/kg dose of the EPO albumin fusion protein encoded by albumin fusion construct 1966 cleared this EPO albumin fusion protein 7 times more slowly than rhEPO.

EXAMPLE 9: Construct ID 1981, HSA-EPO, Generation.

[0938] Construct ID 1981, pC4.HSA-EPO.A28-D192, comprises DNA encoding for an EPO albumin fusion protein which has the HSA full-length sequence, including the native

HSA leader sequence, fused to the amino terminus of the mature form of EPO, with the exception of the final Arg residue, cloned into the mammalian expression vector pC4.

Cloning of EPO cDNA for construct 1981

[0939] The DNA encoding EPO was amplified with primers EPO3 and EPO4, described below, cut with *Bsu* 36I/*Asc* I, and ligated into *Bsu* 36I/*Asc* I cut pC4:HSA. Construct ID #1981 encodes an albumin fusion protein containing the native leader sequence and mature form of HSA and the mature form of EPO, Ala 28 to Asp 192 (Genbank Accession AAA52400).

[0940] Two oligonucleotides suitable for PCR amplification of the polynucleotide encoding the mature form of EPO (see SEQ ID NO:X for construct 1981 in Table 2), EPO3 and EPO4, were synthesized:

EPO3: 5'- AAGCTGCCTTAGGCTTA*GCCCCACCACGCCTCATCTGTGACAG* -3' (SEQ ID NO: 805)

EPO4: 5'- GCGCGGCGCGCCGAATTCCTATTA*GTCCCCTGTCCTGCAGGCCTCCCCTGTG* -3' (SEQ ID NO: 806)

[0941] EPO3 incorporates a *Bsu* 36I cloning site (shown underlined) and nucleotides encoding the last four amino acid residues of the mature form of HSA, as well as 26 nucleotides, italicized, encoding the first 8 amino acid residues of the mature form of EPO. In EPO4, the *Asc* I site is underlined (SEQ ID NO:806) and the last 28 nucleotides, italicized, are the reverse complement of DNA encoding the last 9 amino acid residues of EPO (for general construct cloning see Example 5) , with the exception of the final Arg residue. The PCR amplimer generated using these primers was purified, digested with *Bsu* 36I and *Asc* I restriction enzymes, and cloned into the *Bsu* 36I and *Asc* I sites of the pC4:HSA vector.

[0942] The PCR product was purified (for example, by using Wizard PCR Preps DNA Purification System (Promega Corp)) and then digested with *Bsu36*I and *Asc*I. After further purification of the *Bsu36*I-*Asc*I fragment by gel electrophoresis, the product was cloned into *Bsu36*I/*Asc*I digested pC4:HSA to give construct ID # 1981.

[0943] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing confirmed the presence of the expected HSA sequence (see below).

[0944] EPO albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of EPO lacking the final Arg residue, i.e., Ala-28 to Asp-192. In one embodiment of the invention, EPO albumin fusion proteins of the invention further comprise a signal

sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature EPO albumin fusion protein is secreted directly into the culture medium. EPO albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, EPO albumin fusion proteins of the invention comprise the native EPO signal sequence. In further preferred embodiments, the EPO albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 1981.

Expression in CHO cells.

[0945] Construct 1981 was transfected into CHO cells as described in Examples 6 and 8. Expression levels and the specific productivity rates were determined as described in Example 8.

Purification from CHO supernatant.

[0946] The cell supernatant containing the EPO albumin fusion protein expressed from construct ID #1981 in CHO cells was purified as described in Examples 7 and 8. N-terminal sequencing generated DAHKS, the sequence of the amino terminus of the mature form of HSA. For each litre of supernatant, 14 mg of protein was obtained. An approximate MW of 85.7 kDa was obtained.

In vitro TF-1 cell proliferation assay for construct 1981.

Method

[0947] The *in vitro* TF-1 cell proliferation assay for the EPO albumin fusion protein encoded by construct 1981 was carried out as previously described in Example 8 under subsection heading "*In vitro* TF-1 cell proliferation assay for construct 1966".

Results

[0948] Supernatants from CHO cells expressing construct 1981 were >90% purified for the HSA–EPO albumin fusion protein and were tested in the assay, as described in

Example 8. On average, an EC50 of greater than 5 fold of that of rhEPO was established (see Figures 4 and 7).

The activity of construct 1981 can be assayed using an in vivo Harlan mouse model for measuring hematocrit.

Methods

[0949] The *in vivo* Harlan mouse model was used to assay for hematocrit levels upon subcutaneous administration of either control rhEPO or EPO albumin fusion protein encoded by construct 1981. The assay was carried out as previously described in Example 8 under subsection heading "The activity of construct 1966 can be assayed using an *in vivo* Harlan mouse model for measuring hematocrit".

Results

[0950] There was a significant increase in hematocrit (see Figure 5) from day 0 to day 7 for animals treated with either rhEPO or EPO albumin fusion proteins. However, the EPO albumin fusion protein encoded by construct 1981 appears to have a more drastic effect on hematocrit levels than the rhEPO control.

[0951] DBA mice injected intravenously with a 150 μg/kg dose of EPO albumin fusion protein encoded by albumin fusion construct 1981 cleared this EPO albumin fusion protein 7 times more slowly than rhEPO.

EXAMPLE 10: Construct ID 1997, EPO-HSA, Generation.

[0952] Construct ID 1997, pEE12.1:EPO M1-D192.HSA, comprises DNA encoding an EPO albumin fusion protein which has the full-length EPO protein (including the native leader sequence), i.e., M1-D192, with the exception of the final Arg residue, fused to the amino-terminus of the mature form of HSA cloned into the mammalian expression vector pEE12.1.

Cloning of EPO cDNA for construct 1997.

[0953] The DNA encoding EPO was amplified with primers EPO5 and EPO6, described below, cut with *Eco* RI/*Cla* I, and ligated into *Eco* RI/*Cla* I cut pcDNA3 (Invitrogen Corporation, 1600 Faraday Ave, Carlsbad, CA 92008). pcDNA3.EPO M1-D192.HSA was digested with *Eco* RI/*Hind* III to release the EPO M1-D192.HSA expression cassette fragment and cloned into *Eco* RI/*Hind* III digested pEE12.1. Construct ID #1997

encodes an albumin fusion protein containing the leader sequence and the mature form of EPO, followed by the mature HSA protein (see SEQ ID NO:Y in Table 2 for construct 1997).

[0954] Two oligonucleotides suitable for PCR amplification of the polynucleotide encoding EPO (SEQ ID NO:X, Table 2 for construct 1997), EPO5 and EPO6, were synthesized.

EPO5:5'-GATC*GAATTC*GCCACCATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTTCTCCTGTCC CTGCTGTCGCTCCCTCTGGGCCTCCCAGTCCTGGGCGCCCCACCACGCCTCATCTGTGAC– 3' (SEQ ID NO: 775)

EPO6:5'-CTTTAA*ATCGAT*GAGCAACCTCACTCTTGTGTGCATCGTCCCCTGTCCTGCAGGCCTCCC-3' (SEQ ID NO: 776)

[0955] EPO5 incorporates an *Eco* RI site (shown in italics) and a kozak sequence (shown underlined) prior to the DNA encoding the first 35 amino acids of the ORF of the full-length EPO. In EPO6, the italicized sequence is a *Cla* I site, the underlined sequence is the reverse complement of the DNA encoding the first 9 amino acids of the mature form of HSA protein (DAHKSEVAH, SEQ ID NO:1106), and the sequence following the reverse complement of HSA is the reverse complement of the last 23 nucleotides encoding the last 7 amino acids of EPO not including the final Arg-193 amino acid. Using these two primers, DNA encoding the full-length EPO protein was PCR amplified as in Example 8.

[0956] The PCR product was purified and then digested with *Eco RI* and *Cla* I. After further purification of the *Eco RI-Cla* I fragment by gel electrophoresis, the product was cloned into *Eco RI/Cla* I digested pcDNA3. The *Eco* RI/*Hind* III fragment containing the expression cassette was generated from pcDNA3.EPO.M1-D192.HSA and subcloned into the *Eco* RI/*Hind* III digested pEE12.1 to give construct ID # 1997.

[0957] Further, analysis of the N-terminus of the albumin fusion protein by amino acid sequencing confirmed the presence of the expected EPO sequence (see below).

[0958] EPO albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of EPO lacking the final Arg residue, i.e., Ala-28 to Asp-192. In one embodiment of the invention, EPO albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature EPO albumin fusion protein is secreted directly into the culture medium. EPO albumin fusion proteins of the invention may comprise

heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, EPO albumin fusion proteins of the invention comprise the native EPO signal sequence. In further preferred embodiments, the EPO albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 1997.

Expression in NS0 cells.

[0959] Construct 1997 was transfected into NS0 cells as described in Example 6. Expression levels and specific productivity rates were determined as described in Example 8.

Purification from NS0 cell supernatant.

[0960] Purification of the EPO albumin fusion protein from 500 mL cell supernatant from NS0 cells transfected with construct 1997 involves Q-Sepharose anion exchange chromatography at pH 7.4 using a NaCl gradient from 0 to 1 M in 20 mM Tris-HCl, followed by Poros PI 50 anion exchange chromatography at pH 6.5 with a sodium citrate gradient from 5 to 40 mM, and diafiltrating for 6 DV into 10 mM citrate, pH 6.5 and 140 mM NaCl, the final buffer composition (see, Example 7). N-terminal sequencing yielded the sequence APPRLI which is the amino terminus of the mature form of EPO. The protein has an approximate MW of 87.7 kDa. A final yield of 52.2 mg protein per L of supernatant was obtained.

[0961] For larger scale purification, 50 L of NS0 cell supernatant can be concentrated into ~8 to 10 L. The concentrated sample can then be passed over the Q-Sepharose anion exchange column (10 x 19 cm, 1.5 L) at pH 7.5 using a step elution consisting of 50 mM NaOAc, pH 6.0 and 150 mM NaCl. The eluted sample can then be virally inactivated with 0.75% Triton-X 100 for 60 min at room temperature. SDR-Reverse Phase chromatography (10 cm x 10 cm, 0.8 L) can then be employed at pH 6.0 with 50 mM NaOAc and 150 mM NaCl, or alternatively, the sample can be passed over an SP-sepharose column at pH 4.8 using a step elution of 50 mM NaOAc, pH 6.0, and 150 mM NaCl. DV 50 filtration would follow to remove any viral content. Phenyl-650M chromatography (20 cm x 12 cm, 3.8 L) at pH 6.0 using a step elution consisting of 350 mM $(NH_4)_2SO_4$ and 50 mM NaOAc, or

alternatively consisting of 50 mM NaOAc pH 6.0, can follow. Diafiltration for 6-8 DV will allow for buffer exchange into the desired final formulation buffer of either 10 mM Na_2HPO_4 + 58 mM sucrose + 120 mM NaCl, pH 7.2 or 10 mM citrate, pH 6.5, and 140 mM NaCl.

In vitro TF-1 cell proliferation assay for construct 1997.

Method

[0962] The *in vitro* TF-1 cell proliferation assay for the EPO-HSA albumin fusion encoded by construct 1997 was carried out as previously described in Example 8 under subsection heading "*In vitro* TF-1 cell proliferation assay for construct 1966".

Results

[0963] Supernatants from NS0 cells expressing construct 1997 were >90% purified for the EPO-HSA albumin fusion protein and were tested in the assay, as described in Example 8. On average, an EC50 of greater than 5 fold of that of rhEPO was established (see Figure 7).

The activity of construct 1997 can be assayed using an in vivo Harlan mouse model for measuring hematocrit.

Methods

[0964] The *in vivo* Harlan mouse model was used to assay for hematocrit levels upon subcutaneous administration of either control rhEPO or EPO albumin fusion protein encoded by construct 1981 at various doses on days 0, 2, 4, and 6. The assay was carried out as previously described in Example 8 under subsection heading "The activity of construct 1966 can be assayed using an *in vivo* Harlan mouse model for measuring hematocrit". Hematocrit was determined on days 0, 8, and 14.

Results

[0965] There was a significant and similar increase in hematocrit (see Figure 8) from day 0 to day 8 for animals treated with either rhEPO or the EPO albumin fusion encoded by construct 1997. However, as was the case for the EPO albumin fusion protein encoded by construct 1966 but to a lesser extent, subcutaneous administration of 3 doses/week of 52 μg/kg of EPO albumin fusion encoded by construct 1997 caused close to 30% change in hematocrit from day 0 to day 8 and subdued to ~15% on day 14 (see Figure 6) as opposed to a decline from close to 30% to <10% for a triple dose of 12 μg/kg subcutaneous administration of rhEPO per week.

[0966] DBA mice injected intravenously with a 150 μg/kg dose of EPO-HSA cleared this EPO albumin fusion 7 times more slowly than rhEPO.

EXAMPLE 11: Construct ID 2294, EPO-HSA, Generation.

[0967] Construct ID 2294, pC4.EPO.R140G.HSA, comprises DNA encoding an EPO-HSA fusion protein which has the full-length EPO protein including the native leader sequence of the EPO protein, with the exception of the final Arg residue, i.e., M1-D192, with a point mutation mutating Arg-140 to Gly, fused to the amino-terminus of the mature form of HSA cloned into the mammalian expression vector pC4.

Cloning of EPO cDNA for construct 2294.

[0968] Construct ID #2294 encodes an albumin fusion protein containing the leader sequence and the mature form of EPO, followed by the mature HSA protein. Construct ID #2294 was generated by using construct ID #1966, i.e., pC4:EPO.M1-D192.HSA) as a template in a two-step PCR method.

[0969] Four oligonucleotides suitable for PCR amplification of the polynucleotide encoding EPO (SEQ ID NO:X for construct 2294, table 2), EPO7, EPO8, EPO9, and EPO10, were synthesized.

EPO7: 5'-CTTT*GGATCC*GCCACCATGGGGGTGCACGAATGT(primer 82848)– 3' (SEQ ID NO: 915)

EPO8: 5'-CCTTCTGGGC**TCC**CAGAGCCCGAAG (primer 82847)-3' (SEQ ID NO: 1123)

EPO9: 5'-CATTATCGATGAGCAACCTCACTCTTGTGTGCATCGTCCC (primer 82849)-3' (SEQ ID NO: 916)

EPO10: 5'-CTTCGGGCTCTG**GGA**GCCCAGAAGG (primer 82846)-3' (SEQ ID NO: 1124)

[0970] In the first round of PCR amplifications, the N-terminal and the C-terminal fragments of construct ID 1966 were independently amplified. The N-terminal fragment was generated using primers EPO7 and EPO8. EPO7 incorporates *Bam* HI (shown in italics) and has a kozak sequence (shown underlined) prior to the first 18 nucleotides encoding the first 6 amino acids of the ORF of the full-length EPO. The EPO8 primer comprises the reverse complement of the sequence spanning amino acids 136 to 143 of the full-length form of EPO with the exception that the codon CGA encoding the Arg residue at amino acid 140 (highlighted in bold) is altered to the codon GGA which encodes a Gly residue. The C-terminal fragment was generated using primers EPO9 and EPO10. In EPO9, the underlined sequence is a *Cla* I site; and the *Cla* I site and the DNA following it are the reverse

complement of DNA encoding the first 10 amino acids of the mature HSA protein (SEQ ID NO:1038). In EPO9, the last 5 nucleotides correspond to the reverse complement of the last 5 nucleotides in the full-length EPO, which lacks the final Arg-193 residue. The EPO10 primer comprises the nucleic acid sequence encoding amino acids 136 to 143 of the full-length form of EPO with the exception that the codon CGA encoding the Arg residue at amino acid 140 (highlighted in bold) is altered to the codon GGA which encodes a Gly residue. In the second round of PCR amplifications, primers EPO7 and EPO9 were used to amplify the full-length of EPO with the Arg-140 to Gly mutation in which the reaction mixture contained both the PCR amplified N-terminal fragment and the PCR amplified C-terminal fragment.

[0971] The PCR product was purified and then digested with *Bam* HI and *Cla* I. After further purification of the *Bam* HI-*Cla* I fragment by gel electrophoresis, the product was cloned into *Bam* HI/*Cla* I digested pC4:HSA to give construct ID # 2294.

[0972] Further, analysis of the N-terminus of the albumin fusion protein by amino acid sequencing can confirm the presence of the expected EPO sequence (see below).

[0973] EPO albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of EPO lacking the final Arg residue, i.e., Ala-28 to Asp-192. In one embodiment of the invention, EPO albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature EPO albumin fusion protein is secreted directly into the culture medium. EPO albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, EPO albumin fusion proteins of the invention comprise the native EPO signal sequence. In further preferred embodiments, the EPO albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2294.

Expression in CHO cells.

[0974] Construct 2294 can be transfected into CHO cells as described in Examples 6 and 8. Expression levels and specific productivity rates can be determined as described in Example 8.

Purification from CHO supernatant.

[0975] The cell supernatant containing the EPO-HSA fusion protein expressed from construct ID #2294 in CHO cells can be purified as in Examples 7 and 8. N-terminal sequencing should yield the sequence APPRLI (SEQ ID NO:2141) which corresponds to the amino terminus of the mature form of EPO and should yield a protein of approximate MW of 87.7 kDa.

In vitro TF-1 cell proliferation assay for construct 2294.

Method

[0976] The *in vitro* TF-1 cell proliferation assay for the EPO-HSA albumin fusion encoded by construct 2294 can be carried out as previously described in Example 8 under subsection heading "*In vitro* TF-1 cell proliferation assay for construct 1966".

The activity of construct 2294 can be assayed using an in vivo Harlan mouse model for measuring hematocrit.

[0977] The *in vivo* Harlan mouse model as previously described in Example 8 under subsection heading, "*In vivo* Harlan mouse model for measuring hematocrit", can be used to measure hematocrit levels for the EPO albumin fusion protein encoded by construct 2294.

EXAMPLE 12: Construct ID 2298, EPO-HSA, Generation.

[0978] Construct ID 2298, pEE12.1:EPO.R140G.HSA, comprises DNA encoding an EPO albumin fusion protein which has the full-length EPO protein (including the native leader sequence), with the exception of the final Arg residue, i.e., M1-D192, with a point mutation mutating Arg-140 to Gly, fused to the amino-terminus of the mature form of HSA cloned into the mammalian expression vector pEE12.1.

Cloning of EPO cDNA for construct 2298

[0979] Construct ID #2298 encodes an albumin fusion protein containing the leader sequence and the mature form of EPO, followed by the mature HSA protein. Construct ID

#2298 was generated by using construct ID #1997, i.e., pEE12.1:EPO.M1-D192.HSA) as a template for PCR mutagenesis.

[0980] Two oligonucleotides suitable for PCR amplification of template of construct ID #1997, EPO11 and EPO12, were synthesized.

EPO11: 5'-GGCTTCCTTCTGGGC**TCC**CAGAGCCCGAAGCAG-3' (SEQ ID NO: 924)

EPO12: 5'-CTGCTTCGGGCTCTG**GGA**GCCCAGAAGGAAGCC-3' (SEQ ID NO: 923)

[0981] The EPO11 anti-sense primer comprises the reverse complement of the sequence spanning amino acids 135 to 145 of the full-length form of EPO with the exception that the codon CGA encoding the Arg residue at amino acid 140 (highlighted in bold) is altered to the codon GGA which encodes a Gly residue. The EPO12 sense primer comprises the nucleic acid sequence encoding amino acids 135 to 145 of the full-length form of EPO with the exception that the codon CGA encoding the Arg residue at amino acid 140 (highlighted in bold) is altered to the codon GGA which encodes a Gly residue. Using the Site Directed Mutagenesis kit and protocol from Stratagene, the PCR reaction generated the whole template of construct ID #1997 with the exception of the Arg to Gly mutation. The PCR product was digested with *Dpn* I, transformed into competent XL1 Blue bacteria, and colonies were sequenced and confirmed. The *Dpn* I endonuclease is specific for methylated and hemimethylated DNA and targets the sequence 5'-GmATC-3'. *Dpn* I is used to digest the parental DNA template so as to select the mutation-containing synthesized DNA.

[0982] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing can confirm the presence of the expected EPO sequence (see below).

[0983] EPO albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of EPO lacking the final Arg residue, i.e., Ala-28 to Asp-192. In one embodiment of the invention, EPO albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature EPO albumin fusion protein is secreted directly into the culture medium. EPO albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, EPO albumin fusion proteins

of the invention comprise the native EPO signal sequence. In further preferred embodiments, the EPO albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2298.

Expression in NS0 cells.

[0984] Construct 2298 can be transfected into NS0 cells as described in Examples 6 and 10. Expression levels and specific productivity rates can be determined as described in Example 8.

Purification from NS0 cell supernatant.

[0985] The cell supernatant containing the EPO-HSA fusion protein expressed from ID #2298 in NS0 cells can be purified as in Examples 7 and 10. N-terminal sequencing should yield the sequence APPRLI (SEQ ID NO:2141) which corresponds to the amino terminus of the mature form of EPO and should yield a protein of approximate MW of 87.7 kDa.

In vitro TF-1 cell proliferation assay for construct 2298.

Method

[0986] The *in vitro* TF-1 cell proliferation assay for the EPO-HSA albumin fusion protein encoded by construct 2298 can be carried out as previously described in Example 8 under subsection heading "*In vitro* TF-1 cell proliferation assay for the albumin-fusion protein encoded by construct 1966" and in Example 10 under subsection heading "*In vitro* TF-1 cell proliferation assay for construct 1997".

The activity of construct 2298 can be assayed using an in vivo Harlan mouse model for measuring hematocrit.

[0987] The *in vivo* Harlan mouse model as previously described in Example 8 under subsection heading, "*In vivo* Harlan mouse model for measuring hematocrit", and in Example 10 can be used to measure hematocrit levels for the EPO albumin fusion protein encoded by construct 2298.

EXAMPLE 13: Construct ID 2325, EPO-HSA, Generation.

[0988] Construct ID 2325, pC4.EPO:M1-D192.HSA.codon optimized, comprises DNA encoding an EPO albumin fusion protein which has the full-length EPO protein (including the native leader sequence), i.e., M1-D192 with the Arg-140 to Gly mutation, fused to the amino-terminus of the mature form of HSA cloned into the mammalian expression vector pC4.

Cloning of EPO cDNA for construct 2325

[0989] DNA encoding the EPO open reading frame was codon optimized so as not to hybridize to the wild-type EPO gene sequence. The polynucleotide encoding EPO was PCR generated by 6 overlapping oligonucleotides and cloned into the TA vector. Construct ID #2325 encodes an albumin fusion protein containing the leader sequence and the mature form of EPO, followed by the mature HSA protein.

[0990] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing can confirm the presence of the expected EPO sequence (see below).

[0991] EPO albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of EPO lacking the final Arg residue, i.e., Ala-28 to Asp-192. In one embodiment of the invention, EPO albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature EPO albumin fusion protein is secreted directly into the culture medium. EPO albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, EPO albumin fusion proteins of the invention comprise the native EPO signal sequence. In further preferred embodiments, the EPO albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2325.

Expression in CHO cells.

[0992] Construct 2325 can be transfected into CHO cells as described in Examples 6 and 8. Expression levels and specific productivity rates can be determined as describe in Example 8.

Purification from CHO supernatant.

[0993] The cell supernatant containing the EPO-HSA fusion protein expressed from construct ID #2325 in CHO cells can be purified by methods described in Examples 7 and 8. N-terminal sequencing should yield the sequence APPRLI (SEQ ID NO:2141) which corresponds to the amino terminus of the mature form of EPO and should yield a protein of approximate MW of 87.7 kDa.

In vitro TF-1 cell proliferation assay for construct 2325.

Method

[0994] The *in vitro* TF-1 cell proliferation assay for the EPO-HSA albumin fusion encoded by construct 2325 can be carried out as previously described in Example 8 under subsection heading "*In vitro* TF-1 cell proliferation assay for construct 1966".

The activity of construct 2325 can be assayed using an in vivo Harlan mouse model for measuring hematocrit.

[0995] The *in vivo* Harlan mouse model as previously described in Example 8 under subsection heading, "*In vivo* Harlan mouse model for measuring hematocrit", can be used to measure hematocrit levels for the EPO albumin fusion protein encoded by construct 2325.

EXAMPLE 14: Indications for EPO Albumin Fusion Proteins.

[0996] Results from *in vitro* and *in vivo* assays described above indicate that EPO albumin fusion proteins can be used in the treatment of bleeding disorders and anemia caused by a variety of conditions, including but not limited to: end-stage renal disease (dialysis patients), chronic renal failure in pre-dialysis, zidovudine-treated HIV patients, cancer patients on chemotherapy, and premature infants. EPO albumin fusion proteins can also be used pre-surgery in anemic patients undergoing elective non-cardiac, non-vascular surgery to reduce the need for blood transfusions. Indications in development for these agents include: aplastic and other refractory anemias, refractory anemia in Inflammatory Bowel Disease, and

transfusion avoidance in elective orthopedic surgery. Anemia in renal disease and oncology are the two primary indications for EPO albumin fusion proteins encoded by constructs 1966, 1981, 1997, 2294, 2298, and 2325.

EXAMPLE 15: Construct ID 1812, IL2-HSA, Generation.

[0997] Construct ID 1812, pSAC35:IL2.A21-T153.HSA, comprises DNA encoding an IL2 albumin fusion protein which has an HSA chimeric leader sequence, i.e., the HSA-kex2 signal peptide, the mature IL2 protein, i.e., A21-T153, fused to the amino-terminus of the mature form of HSA in the yeast *S. cerevisiae* expression vector pSAC35.

Cloning of IL2 cDNA

[0998] The polynucleotide encoding IL2 was PCR amplified using primers IL2-1 and IL2-2, described below. The amplimer was cut with *Sal I/Cla* I, and ligated into *Xho I/Cla* I cut pScCHSA. Construct ID #1812 encodes an albumin fusion protein containing the chimeric leader sequence of HSA, the mature form of IL2, followed by the mature HSA protein.

[0999] Two oligonucleotides suitable for PCR amplification of the polynucleotide encoding the mature form of IL2, IL2-1 and IL2-2, were synthesized:

IL2-1: 5'-AGGAGCGTCGACAAAAGAGCACCTACTTCAAGTTCTACAAAG-3' (SEQ ID NO: 725)

IL2-2: 5'-CTTTAAATCGATGAGCAACCTCACTCTTGTGTGCATCAGTCAGTGTTG AGATGATGCTTTG-3' (SEQ ID NO: 726)

[1000] IL2-1 incorporates the *Sal* I cloning site (shown underlined), nucleotides encoding the last three amino acid residues of the HSA chimeric leader sequence, as well as 24 nucleotides encoding the first 8 amino acid residues of the mature form of IL2. In IL2-2, the *Cla* I site (shown underlined) and the DNA following it are the reverse complement of the DNA encoding the first 10 amino acids of the mature HSA protein (SEQ ID NO:1038) and the last 24 nucleotides are the reverse complement of DNA encoding the last 8 amino acid residues of IL2 (see Example 2). A PCR amplimer of IL2-HSA was generated using these primers, purified, digested with *Sal* I and *Cla* I restriction enzymes, and cloned into the *Xho* I and *Cla* I sites of the pScCHSA vector. After the sequence was confirmed, the expression cassette encoding this IL2 albumin fusion protein was subcloned into pSAC35 as a *Not* I fragment.

[1001] Further, analysis of the N-terminus of the expressed albumin fusion protein by

amino acid sequencing can confirm the presence of the expected IL2 sequence (see below).

[1002] IL2 albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of IL2, i.e., Ala-21 to Thr-153. In one embodiment of the invention, IL2 albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature IL2 albumin fusion protein is secreted directly into the culture medium. IL2 albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, IL2 albumin fusion proteins of the invention comprise the native IL2 signal sequence. In further preferred embodiments, the IL2 albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 1812.

Expression in yeast S. cerevisiae.

[1003] Transfection of construct 1812 into yeast *S. cerevisiae* strain BXP10 was carried out by methods known in the art (see Example 3). Cells were collected at stationary phase after 72 hours of growth. Supernatants from yeast transfected by construct 1812 were collected by clarifying cells at 3000g for 10 min. Expression levels were examined by immunoblot detection with anti-HSA serum (Kent Laboratories) as the primary antibody. An IL2 albumin fusion protein of approximate molecular weight of 85 kDa was obtained. The specific productivity rates were determined via ELISA in which the capture antibody was the US Biological #A1327-35 monoclonal anti-HSA antibody or a monoclonal anti-human IL2 antibody (e.g., from Biosource #AHCO422, Pharmingen #555051, R&D Systems #MAB202, or R&D Systems #MAB602), the detecting antibody was a monoclonal anti-human IL2-biotinylated antibody (e.g., from Biosource #AHC069 or Endogen/Pierce #M-600-B) or a monoclonal anti-HSA antibody Biotrend #4T24, respectively, the conjugate was horseradish peroxidase/streptavidin (Vector Laboratories, #SA-5004), and the substrate was KPL TMB Peroxidase Substrate (KPL #50-76-01). The analysis was carried out according to

manufacturers' protocol and/or by methods known in the art.

Purification from yeast S. cerevisiae cell supernatant.

[1004] The cell supernatant containing IL2 albumin fusion protein expressed from construct ID #1812 in yeast *S. cerevisiae* cells was purified either small scale over a Dyax peptide affinity column (see Example 4) or large scale by following 5 steps: diafiltration, anion exchange chromatography using DEAE-Sepharose Fast Flow column, hydrophobic interaction chromatography (HIC) using Butyl 650S column, cation exchange chromatography using an SP-Sepharose Fast Flow column or a Blue-Sepharose chromatography, and high performance chromatography using Q-sepharose high performance column chromatography (see Example 4). The IL2 albumin fusion protein eluted from the DEAE-Sepharose Fast Flow column with 100 – 250 mM NaCl, from the SP-Sepharose Fast Flow column with 150 – 250 mM NaCl, and from the Q-Sepharose High Performance column at 5 – 7.5 mS/cm. N-terminal sequencing should yield the sequence APTSSST which corresponds to the amino terminus of the mature form of IL2.

The activity of IL2 can be assayed using an in vitro T and NK cell-line proliferation assay.

[1005] The murine CTLL T cell-line is used and is completely dependent on IL2 for cell growth and survival. This cell-line expresses high levels of high affinity IL2 receptors and is extremely sensitive to very low doses of IL2.

Methods

[1006] CTLL-2 cells (murine IL2 dependent T cell-line) is grown in RPMI 10% FBS containing 5 ng/mL recombinant human IL2 and BME. Prior to the assays, the cells are washed twice in PBS to remove IL2. 1 x 10^4 cells/well are seeded in a 96-well plate, in a final volume of 200 µl of RPMI 10% FBS. The yeast and 293T supernatants are tested at final concentrations of : 10%, 5%, and 1%. In addition, recombinant human IL2, "rhIL2", is diluted in the negative control supernatant (HSA alone) to test for the effect of the medium on the stability of the recombinant protein. The cells are cultured at 37 °C for 20 hours, then pulsed with 1 µCi ^{3}H-thymidine for 6 hours. Proliferation is measured by thymidine incorporation, each sample is tested in triplicate.

The activity of the IL2 albumin fusion protein encoded by construct 1812 can be assayed using an in vitro T and NK cell-line proliferation assay.

Methods

[1007] CTLL-2 cells (murine IL2 dependent T cell-line) was grown in RPMI 10% FBS containing 5 ng/mL recombinant human IL2 and BME. Prior to the assays, the cells were washed twice in PBS to remove IL2. 1 x 10^4 cells/well were seeded in a 96-well plate, in a final volume of 200 µl of RPMI 10% FBS. The yeast and 293T supernatants were tested at final concentrations of : 10%, 5%, and 1%. In addition, recombinant human IL2, "rhIL2", was diluted in the negative control supernatant (HSA alone) to test for the effect of the medium on the stability of the recombinant protein. The cells were cultured at 37 °C for 20 hours, then pulsed with 1 µCi ^{3}H-thymidine for 6 hours. Proliferation was measured by thymidine incorporation, each sample was tested in triplicate.

Results

[1008] The IL2 albumin fusion construct ID #1812 stimulated CTLL-2 cell proliferation in a dose-dependent manner (see Figure 9).

The activity of the IL2 albumin fusion protein encoded by construct 1812 can be assayed using an in vivo BALB/c model: RENCA tumor response to therapy.

[1009] The mouse model employs the RENCA adenocarcinoma of BALB/c mice. The RENCA tumor used in these studies arose spontaneously. The RENCA tumors were originally isolated by Dr. Sarah Stewart at the NCI (Bethesda, MD). RENCA tumors grow progressively following transfer of as few as 50 viable cells and spontaneously metastasize from intrarenal implant to the regional lymph nodes, lungs, liver, and spleen, as well as other organs. The immunogenicity of RENCA has been determined to be low to moderate. RENCA bearing mice routinely die within 35-40 days after intrarenal injection of 1 x 10^5 RENCA tumor cells. Mice given RENCA tumor cells intraperitoneally of a similar number of cells usually die within 30-50 days.

Methods

[1010] BALB/c mice (6 – 8 weeks of age) (n=10) were injected subcutaneously in mid-flank with 10^5 RENCA cells obtained from the fourth in vivo passage. After 10 days of daily (QD) or every other day (QOD) injections with placebo (PBS), HSA, rhIL2 at a dose of 0.122 mg/kg/QD or at 200,000 or 300,000 U/mouse, or IL2 albumin fusion protein at 0.61 mg/kg, mice were monitored for change in tumor size at days 14, 17, 21, 25, 28, and 31 post tumor inoculation. The data are presented in dot-analysis where each dot respresents single animals. The horizontal line in each group represents MEAN value (see Figure 10).

Results

[1011] IL2 albumin fusion protein encoded by construct ID#1812 was tested in the above assay.

[1012] Administration of IL2 albumin fusion protein expressed from construct ID#1812 everyday or every other day showed significant impact on tumor growth causing delay of growth and/or shrinkage of tumor size. Every other day administration was more beneficial since tolerance levels were greater (see Figure 10). By day 31 from the inoculation day, 3 mice receiving IL2 albumin fusion products out of 10 were tumor free, only 2 showed signs of reduced tumor, and 4 mice had small tumors that appeared to be shrinking. Only one mouse did not respond beneficially to this treatment. Daily treatment with IL2 albumin fusion protein also caused a delay of growth or actual shrinkage of tumor (2 out of 10 mice were tumor free, 7 remaining mice had small tumors, and 2 had larger ones on the day of experiment termination). All animals receiving IL2 albumin fusion at 0.61 mg/kg were alive on the termination date, while only 40% of the mice receiving placebo (PBS) and 70% of mice receiving HSA were alive. The biological effect was far more pronounced than the recombinant human IL2 given daily either at 200,000 or 300,000 U/mouse. Recombinant human IL2 had only mediocre effect on tumor growth (all mice that received rhIL2 developed tumors and the only effect observed was growth delay) Of the 10 mice receiving rhIL2 (200,000 or 300,000 U/mL), 3 were dead by day 31. The low dose of 0.122 mg/kg/day tested did not inhibit the tumor growth nor spare mice from tumor-related death. The IL2 albumin fusion protein potently inhibited the *in vivo* RENCA growth and caused in several cases full recovery from tumors.

EXAMPLE 16: Construct ID 2030, IL2-HSA, Generation.

[1013] Construct ID 2030, pSAC35:ycoIL2.A21-T153.HSA, comprises DNA encoding an IL2 albumin fusion protein which has the HSA chimeric leader sequence, i.e., the HSA-kex2 signal peptide, the mature form of the IL2 protein, i.e., A21-T153, fused to the amino-terminus of the mature form of HSA in the yeast *S. cerevisiae* expression vector pSAC35.

Cloning of IL2 cDNA

[1014] The IL2 open reading frame "ORF" DNA was codon optimized so as not to hybridize to the wild-type IL2 gene. The polynucleotide encoding the codon optimized IL2 was PCR generated by 6 overlapping oligonucleotides and cloned into a TA vector. The

polynucleotide encoding the codon optimized IL2 was PCR amplified from this clone using primers IL2-3 and IL2-4, described below, cut with *Sal* I/*Cla* I, and ligated into *Xho* I/*Cla* I cut pScCHSA. Construct ID #2030 encodes an albumin fusion protein containing the chimeric leader sequence of HSA and the mature form of IL2 fused to the amino terminus of the mature form of HSA.

[1015] Two oligonucleotides suitable for PCR amplification of the codon optimized polynucleotide encoding the mature form of IL2, IL2-3 and IL2-4, were synthesized:

IL2-3: 5'- AGGAGCGTCGACAAAAGAGCTCCAACTTCTTCTTCTACTAAG-3' (SEQ ID NO: 831)

IL2-4: 5'-CTTTAAATCGATGAGCAACCTCACTCTTGTGTGCATCTGTCAAAGTA GAAATAATAGA TTGGCAG-3' (SEQ ID NO: 832)

[1016] IL2-3 incorporates the *Sal* I cloning site (shown underlined) and encodes for the last three amino acid residues of the chimeric leader sequence of HSA, as well as the 24 nucleotides encoding the first 8 amino acid residues of the mature form of IL2. In IL2-4, the *Cla* I site (shown underlined) and the DNA following it are the reverse complement of the DNA encoding the first 10 amino acids of the mature HSA protein (SEQ ID NO:1038) and the last 24 nucleotides are the reverse complement of DNA encoding the last 8 amino acid residues of IL2 (see Example 2). A PCR amplimer was generated using these primers, purified, digested with *Sal* I and *Cla* I restriction enzymes, and cloned into the *Xho* I and *Cla* I sites of the pScCHSA vector. After the sequence was confirmed, the *Not* I fragment containing the IL2 albumin fusion protein expression cassette was subcloned into pSAC35 cut with *Not* I.

[1017] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing can confirm the presence of the expected IL2 sequence (see below).

[1018] IL2 albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of IL2, i.e., Ala-21 to Thr-153. In one embodiment of the invention, IL2 albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature IL2 albumin fusion protein is secreted directly into the culture medium. IL2 albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein

4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, IL2 albumin fusion proteins of the invention comprise the native IL2 signal sequence. In further preferred embodiments, the IL2 albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2030.

Expression in yeast S. cerevisiae.

[1019] Transfection into yeast *S. cerevisiae* strain BXP10 can be carried out by methods known in the art (see Example 3) and as previously described for construct ID 1812 (see Example 15).

Purification from yeast S. cerevisiae cell supernatant.

[1020] The cell supernatant containing IL2-HSA expressed from construct ID #2030 in yeast *S. cerevisiae* cells can be purified either small scale over a Dyax peptide affinity column (see Example 4) or large scale by following 5 steps: diafiltration, anion exchange chromatography using DEAE-Sepharose Fast Flow column, hydrophobic interaction chromatography (HIC) using Butyl 650S column, cation exchange chromatography using an SP-Sepharose Fast Flow column or a Blue-Sepharose chromatography, and high performance chromatography using Q-sepharose high performance column chromatography (see Example 4 and Example 15). N-terminal sequencing should yield the sequence APTSSST (SEQ ID NO:2142) which corresponds to the amino terminus of the mature form of IL2.

The activity of the IL2 albumin fusion protein encoded by construct 2030 can be assayed using the in vitro T and NK cell-line proliferation assay.

[1021] The activity of construct ID 2030 can be assayed using an *in vitro* T and NK cell-line proliferation assay as in Example 15 .

The activity of the IL2 albumin fusion protein encoded by construct 2030 can be assayed using an in vivo BALB/c model: RENCA tumor response to therapy.

[1022] The activity of the IL2 albumin fusion protein encoded by construct 2030 can be assayed using the *in vivo* BALB/c model as described in Example 15 in which the RENCA tumor response to therapy is monitored.

EXAMPLE 17: Construct ID 2031, HSA-IL2, Generation.

[1023] Construct ID 2031, pSAC35:HSA.ycoIL2.A21-T153, comprises DNA encoding an IL2 albumin fusion protein which has the HSA full-length sequence that includes the HSA chimeric leader sequence, i.e., the HSA-kex2 signal peptide, fused to the amino-terminus of the mature form of IL2, A21-T153, in the yeast *S. cerevisiae* expression vector pSAC35.

Cloning of IL2 cDNA

[1024] The IL2 open reading frame "ORF" DNA was codon optimized so as not to hybridize to the wild-type IL2 gene. The polynucleotide encoding the codon optimized IL2 was PCR generated by 6 overlapping oligonucleotides and cloned into a TA vector. The polynucleotide encoding the codon optimized IL2 was PCR amplified from this clone using primers IL2-5 and IL2-6, described below, cut with *Bsu* 36I/*Pme* I, and ligated into *Bsu* 36I/*Pme* I cut pScNHSA. Construct ID #2031 encodes an albumin fusion protein containing the chimeric leader sequence and mature form of HSA and the mature form of IL2.

[1025] Two oligonucleotides suitable for PCR amplification of the codon optimized polynucleotide encoding the mature form of IL2, IL2-5 and IL2-6, were synthesized:

IL2-5: 5'-AAGCTGCCTTAGGCTTAGCTCCAACTTCTTCTTCTACTAAG-3' (SEQ ID NO: 833)

IL2-6: 5'-GCGCGCGTTTAAACGGTACCTTATGTCAAAGTAGAAATAATAGATTGG CAG-3' (SEQ ID NO:834)

[1026] IL2-5 incorporates the *Bsu* 36I cloning site (shown underlined) and encodes for the last four amino acid residues of the mature form of HSA, as well as the 24 nucleotides encoding the first 8 amino acid residues of the mature form of IL2. In IL2-6, the *Pme* I site is underlined (SEQ ID NO:834) and the last 24 nucleotides are the reverse complement of DNA encoding the last 8 amino acid residues of IL2 (see Example 2). A PCR amplimer was generated using these primers, purified, digested with *Bsu* 36I and *Pme* I restriction enzymes, and cloned into the *Bsu* 36I and *Pme* I sites of the pScNHSA vector. After the sequence was confirmed, the *Not* I fragment containing the IL2 albumin fusion protein expression cassette was subcloned into pSAC35 cut with *Not* I.

[1027] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing can confirm the presence of the expected HSA sequence (see below).

[1028] IL2 albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of IL2, i.e., Ala-21 to Thr-153. In one embodiment of the invention, IL2 albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature IL2 albumin fusion protein is secreted directly into the culture medium. IL2 albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, IL2 albumin fusion proteins of the invention comprise the native IL2 signal sequence. In further preferred embodiments, the IL2 albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2031.

Expression in yeast S. cerevisiae.

[1029] Transfection into yeast *S. cerevisiae* strain BXP10 can be carried out by methods known in the art (see Example 3) and as previously described for construct ID 1812 (see Example 15).

Purification from yeast S. cerevisiae cell supernatant.

[1030] The cell supernatant containing HSA-IL2 expressed from construct ID #2031 in yeast *S. cerevisiae* cells can be purified either small scale over a Dyax peptide affinity column (see Example 4) or large scale by following 5 steps: diafiltration, anion exchange chromatography using DEAE-Sepharose Fast Flow column, hydrophobic interaction chromatography (HIC) using Butyl 650S column, cation exchange chromatography using an SP-Sepharose Fast Flow column or a Blue-Sepharose chromatography, and high performance chromatography using Q-sepharose high performance column chromatography (see Example 4 and Example 15). N-terminal sequencing should yield the sequence DAHKS (SEQ ID NO:2143) which corresponds to the amino terminus of the mature form of HSA.

The activity of the IL2 albumin fusion protein encoded by construct 2031 can be assayed

using the in vitro T and NK cell-line proliferation assay.

[1031] The activity of construct ID 2031 can be assayed using an *in vitro* T and NK cell-line proliferation assay described in Example 15 .

The activity of the IL2 albumin fusion protein encoded by construct 2031 can be assayed using the in vivo BALB/c model: RENCA tumor response to therapy.

[1032] The activity of the IL2 albumin fusion protein encoded by construct 2031 can be assayed using the *in vivo* BALB/c model as described in Example 15 in which the RENCA tumor response to therapy is monitored.

EXAMPLE 18: Indications for IL2 Albumin Fusion Proteins.

[1033] Indications for IL2 albumin fusion proteins (including, but not limited to, those encoded by constructs 1812, 2030, and 2031) include, but are not limited to, solid tumors, metastatic renal cell carcinoma, metastatic melanoma, malignant melanoma, renal cell carcinoma, HIV infections treatment (AIDS), inflammatory bowel disorders, Kaposi's sarcoma, leukemia, multiple sclerosis, rheumatoid arthritis, transplant rejection, type I diabetes mellitus, lung cancer, acute myeloid leukemia, hepatitis C, non-Hodgkin's Lymphoma, and ovarian cancer.

EXAMPLE 19: Construct ID 1642, GCSF-HSA, Generation.

[1034] Construct ID 1642, pSAC35:GCSF.T31-P204.HSA, comprises DNA encoding a GCSF albumin fusion protein which has the HSA chimeric leader sequence, i.e., the HSA-kex2 signal peptide, the mature form of the "short form" of Granulocyte Colony Stimulating Factor, "G-CSF", protein, i.e., T31-P204, fused to the amino-terminus of the mature form of HSA in the yeast *S. cerevisiae* expression vector pSAC35.

Cloning of GCSF cDNA

[1035] A polynucleotide encoding GCSF was PCR amplified using primers GCSF-1 and GCSF-2, described below. The amplimer was cut with *Sal I/Cla* I, and ligated into *Xho I/Cla* I cut pScCHSA. Construct ID #1642 comprises DNA which encodes an albumin fusion protein containing the chimeric leader sequence of HSA, the mature form of GCSF, followed by the mature HSA protein.

[1036] Two oligonucleotides suitable for PCR amplification of a polynucleotide

encoding the mature form of GCSF, GCSF-1 and GCSF-2, were synthesized:

GCSF-1: 5'- GAATTCGTCGACAAAAGAACCCCCCTGGGCCCTGCCAG -3' (SEQ ID NO:665)

GCSF-2: 5'-AAGCTTATCGATGAGCAACCTCACTCTTGTGTGCATCGGGCTGGGC AAGGTGGCGTAG-3' (SEQ ID NO:666)

[1037] GCSF-1 incorporates the *Sal* I cloning site (shown underlined), nucleotides encoding the last three amino acid residues of the HSA chimeric leader sequence, as well as 20 nucleotides encoding the first 6 amino acid residues of the mature form of GCSF. In GCSF-2, the *Cla* I site (shown underlined) and the DNA following it are the reverse complement of the DNA encoding the first 10 amino acids of the mature HSA protein (SEQ ID NO:1038) and the last 21 nucleotides are the reverse complement of DNA encoding the last 7 amino acid residues of GCSF. Using these primers, a PCR amplimer was generated, purified, digested with *Sal* I and *Cla* I restriction enzymes, and cloned into the *Xho* I and *Cla* I sites of the pScCHSA vector. After the sequence was confirmed, the *Not* I fragment containing the GCSF albumin fusion expression cassette was subcloned into pSAC35 cut with *Not* I.

[1038] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing confirmed the presence of the expected GCSF sequence (see below).

[1039] GCSF albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of GCSF, i.e., Thr-31 to Pro-204. In one embodiment of the invention, GCSF albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature GCSF albumin fusion protein is secreted directly into the culture medium. GCSF albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, GCSF albumin fusion proteins of the invention comprise the native GCSF signal sequence. In further preferred embodiments, the GCSF albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by

the invention.

Expression and Purification of Construct ID 1642.

Expression in yeast S. cerevisiae.

[1040] Transformation of construct 1642 into yeast *S. cerevisiae* strains D88, BXP10, and DXY1 – a YAP3 mutant, was carried out by methods known in the art (see Example 3). A preliminary "Halo Assay" was carried out to assess if the transformed yeast are producing the proteins encoded by the fusion constructs. Secretion of HSA fusion proteins into agar media containing anti-HSA antibodies will result in the formation of an insoluble "precipitin" ring or halo. The size of the halo is proportional to the amount of HSA protein being produced. LEU2 + prototrophs were selected on synthetic complete leucine dropout medium containing dextrose, "SCD-Leu". Selected colonies as well as a positive control were gridded onto a BMMD plate containing anti-HSA antibody. After growth, the plates were incubated at 4°C to allow for precipitin ring formation. Based on the "Halo Assay", colonies from transformation of construct 1642 produced protein. To establish the extent of secretion, transformed cells were collected at stationary phase after 48 hours of growth in suspension. Supernatants were collected by clarifying cells at 3000g for 10 min. Expression levels were examined by immunoblot detection with anti-HSA serum (Kent Laboratories) or with an antibody directed to the Therapeutic protein portion, i.e., GCSF, of the albumin fusion protein. The GCSF albumin fusion protein of approximate molecular weight of 88 kDa was obtained. To obtain workable quantities for purification, the yeast transformants were inoculated in 1 L of BMM media at 150 rpm, 29.5°C. The culture was centrifuged and passed through a 0.45 □m filter. The specific productivity rates can be determined via ELISA in which, for example, the capture antibody is the R&D Systems Clone 3316.111 monoclonal mouse anti-GCSF, the detecting antibody is the R&D Systems BAF214 (i.e., Clone ACN030081) biotinylated goat anti-human GCSF antibody, the conjugate is horseradish peroxidase/streptavidin (Vector Laboratories, #SA-5004), and the substrate is KPL TMB Peroxidase Substrate (KPL #50-76-01), where the analysis is carried out according to manufacturers' protocol and/or by methods known in the art.

Purification from yeast S. cerevisiae cell supernatant.

[1041] A general purification procedure for albumin fusion proteins has been described in Example 4. The purification of GCSF albumin fusion protein is described specifically below. Another purification scheme is described in Example 20.

Step 1: Phenyl Fast Flow Chromatography (Amersham Pharmacia Biotech)

[1042] The yeast culture supernatant (3 L) containing GCSF-HSA encoded by construct 1642 was loaded onto a phenyl fast flow column with 1 M of ammonium sulfate in 50 mM Tris, pH 7.2. The column was washed with 1 M of ammonium sulfate in 50 mM Tris, pH 7.2, 0.2 M ammonium sulfate in 50 mM Tris, pH 7.2, and then washed with the buffer. The GCSF-HSA fusion protein was eluted with water (Water For Injection distilled water WFI).

Step 2: SP Fast Flow Chromatography (Amersham Pharmacia Biotech)

[1043] The eluate of Step 1 was mixed with an equal volume of a solution composed of 10.3 mM Na_2HPO_4 and 4.85 mM citric acid, pH 5.0. The mixture was loaded at 300 cm/hr onto a SP fast flow column and eluted with a solution composed of 0.5 M NaCl in 10.3 mM Na_2HPO_4 and 4.85 mM citric acid, pH 5.0. The column was then stripped with a solution composed of 1M NaCl in 10.3 mM Na_2HPO_4 and 4.85 mM citric acid, pH 5.0.

Step 3: Methyl HIC Chromatography(BioRad)

[1044] The eluate of Step 2 was titrated to a final concentration of 1 M ammonium sulfate (143 mS) in 50 mM Tris, pH 7.2 and loaded onto methyl HIC column. The column was washed to a baseline, then washed with 0.6 M ammonium sulfate in 50 mM Tris, pH 7.2. A gradient from 0.6 M ammonium sulfate to 0 M ammonium sulfate was initiated. The column was finally stripped with WFI and 0.5 M NaOH. A lot of the impurities in the sample eluted at the lower ammonium sulfate concentrations thereby affording the GCSF-HSA fusion high purity.

Step 4: CM Fast Flow Chromatography (Amersham Pharmacia Biotech)

[1045] The eluate of Step 3 was diluted with WFI to 5 mS, pH 5.5 and was loaded onto the CM column at 300 cm/hr. The column was eluted with 0.5 M NaCl in 11 mM Na_2HPO_4 and 4 mM citric acid, pH 5.5. The column was stripped with 1 M NaCl in 11 mM Na_2HPO_4 and 4 mM citric acid, pH 5.5.

Step 5: Ultrafiltration/Diafiltration (Amersham Pharmacia Biotech)

[1046] The purified product was ultrafiltered and diafiltered into Phosphate Buffered *Sal* ine, "PBS", pH 7.2.

[1047] The purified GCSF albumin fusion protein encoded by construct 1642 was analyzed for purity on SDS/PAGE. It was > 95% pure. The protein was sequenced confirmed and also showed 90% purity on N-terminal sequencing with an N-terminal sequence of "TPLGP" (SEQ ID NO:2144).

The activity of GCSF can be assayed using an in vitro NFS-60 cell proliferation assay.

Method

[1048] To assess GCSF activity, NSF-60 cells, a myeloid factor-dependent cell-line derived from Primary Lake Cascitus wild ecotropic virus-induced tumor of NFS mice, are employed.

Cell growth and Preparation

[1049] Cells are originally seeded in T-75 cm^2 flasks at approximately 1.5 x 10^4 cells/mL in growth media (RPMI 1640 containing 10% Fetal Bovine Serum, "FBS", 1x Penicillin/Streptomycin, 1x L-Glutamine (final concentration of 2 mM), and recombinant murine interleukin-3, (IL3) at 30 ng/mL). Cells are split anywhere from 1:10 to 1:20 every 2 days and reseeded in fresh medium.

NFS-60 Bioassay

[1050] The NFS-60 assay is performed as described in Weinstein *et al.* (Weinstein *et al.*, 1986, Proc. Natl. Acad. Sci. U S A, 83, pp5010-4). Briefly, the day before the assay is to be performed, cells are reseeded to 1.0 x 10^5 in fresh assay growth medium containing IL3. The next day cells are transferred to 50 mL conical tubes, centrifuged at low speeds, and washed twice in plain RPMI without serum or growth factors. The pellet is resuspended in 25 mL and the cells are subsequently counted. The cells are spun once more and resuspended at the working concentration in growth medium (described above) but lacking IL3. The cells are plated in 96-well round-bottom TC-treated plates at 1 x 10^5 cells/well. Increasing doses of GCSF are added to each well to a final volume of 0.1 mL. The assay is done in triplicate. The cells are cultured for 24 hours to determine the level of cell proliferation. ^{3}H-Thymidine (5 μCi/mL) is added 4 hours prior to the experiment termination. The cells are then harvested on glass fiber filters using a cell harvester and the amount of ^{3}H-Thymidine labeled DNA is counted using TOP-Count.

The activity of GCSF albumin fusion encoded by construct ID # 1642 can be assayed using an in vitro NFS-60 cell proliferation assay.

Method

[1051] GCSF albumin fusion protein encoded by construct 1642 was tested in the *in vitro* NFS-60 cell proliferation bioassay described above.

Cell growth and Preparation

[1052] Cells were prepared as described above.

NFS-60 Bioassay

[1053] The day before the assay was performed, cells were reseeded to 1.0 x 10^5 in fresh assay growth medium containing IL3. The next day cells were transferred to 50 mL conical tubes, centrifuged at low speeds, and washed twice in plain RPMI without serum or growth factors. The pellet was resuspended in 25 mL and the cells were subsequently counted. The cells were spun once more and resuspended at the working concentration in growth medium (described above) but lacking IL3. The cells were plated in 96-well round-bottom TC-treated plates at 1 x 10^5 cells/well. Increasing doses either of HSA, recombinant human GCSF (rhGCSF), or a partially purified GCSF albumin fusion protein from the yeast supernatant, were added to individual wells to a final volume of 0.1 mL. The assay was done in triplicate. The cells were cultured for 24 hours to determine the level of cell proliferation. ^{3}H-Thymidine (5 □Ci/mL) was added 4 hours prior to the experiment termination. The cells were then harvested on glass fiber filters using cell harvester and the amount of ^{3}H-Thymidine labeled DNA was counted using TOP-Count.

Results

[1054] Construct 1642 demonstrated NFS-60 cell proliferation activity in a dose dependent manner, while the control supernatant from yeast expressing HSA alone did not produce any activity (see Figure 11).

The activity of GCSF can be assayed in vivo using C57BL/6 mice:
GCSF as a Mobilizing Agent.

[1055] G-CSF is capable of mobilizing granulocytes to the periphery as well as increasing the total White Blood Cell, (WBC), count when administered to mice. Recombinant human GCSF, (rhGCSF), cross-reacts with recombinant murine GCSF, (rmGCSF).

Methods

[1056] Mice are ear tagged before the injections start. Mice are injected

intraperitoneally with rhGCSF (Neupogen, AMEN) at either 5 □g (n=5) or 10 □g (n=5) twice a day for 7 consecutive days. The control mice (n=3) receive Hepes Buffered *Sal* ine Solution, (HBSS). At 24 hours after the last rhGCSF administration, peripheral blood is drawn from the tail and analysed for the granulocyte content and total WBC count.

Results

[1057] Both doses of rhGCSF efficiently increase both the frequency and the total number of granulocytes as well as the total WBC count (see Figure 12). This effect is apparent after 24 hours of the final rhGCSF intraperitoneal administration. This effect is transient and the number of granulocytes return to normal values by day 5.

The activity of GCSF albumin fusion protein encoded by construct ID # 1642 can be assayed in vivo using C57BL/6 mice: GCSF-HSA as a Mobilizing Agent.

Methods

[1058] The GCSF albumin fusion protein encoded by construct 1642 can be assayed according to the procedure described above. Briefly, mice are to be ear tagged before the injections are to begin. Mice are to be injected intraperitoneally with either rhGCSF, as a control, or the GCSF albumin fusion protein at either 5 □g (n=5) or 10 □g (n=5) twice a day for 7 consecutive days. Additional control mice (n=3) are to receive Hepes Buffered saline Solution, "HBSS". At 24 hours after the last GCSF administration, peripheral blood can be drawn from the tail and analysed for the granulocyte content and total WBC count.

EXAMPLE 20: Construct ID 1643, HSA-GCSF, Generation.

[1059] Construct ID 1643, pSAC35:HSA.GCSF.T31-P204, comprises DNA encoding a GCSF albumin fusion protein which has the full-length HSA protein that includes the HSA chimeric leader sequence, i.e., the HSA-kex2 signal peptide, fused to the amino-terminus of the mature form of the GCSF protein, i.e., A21-T153, in the yeast *S. cerevisiae* expression vector pSAC35.

Cloning of GCSF cDNA

[1060] The polynucleotide encoding GCSF was PCR amplified using primers GCSF-3and GCSF-4, described below. The amplimer was cut with *Bsu* 36I/*Asc* I, and ligated into *Bsu* 36I/*Asc* I cut pScNHSA. Construct ID #1643 encodes an albumin fusion protein

containing the chimeric leader sequence and mature form of HSA and the mature form of GCSF.

[1061] Two oligonucleotides suitable for PCR amplification of the polynucleotide encoding the mature form of GCSF, GCSF-3 and GCSF-4, were synthesized:

GCSF-3: 5'-AAGCTGCCTTAGGCTTAACCCCCCTGGGCCCTGCCAG -3' (SEQ ID NO:667)

GCSF-4: 5'-GCGCGCGGCGCGCCTCAGGGCTGGGCAAGGTGGCGTAG-3' (SEQ ID NO:668)

[1062] GCSF-3 incorporates the *Bsu* 36I cloning site (shown underlined) and nucleotides encoding the last four amino acid residues of the mature form of HSA, as well as 20 nucleotides encoding the first 6 amino acid residues of the mature form of GCSF. In GCSF-4, the *Asc* I site is underlined and the last 24 nucleotides are the reverse complement of DNA encoding the last 8 amino acid residues of GCSF. A PCR amplimer of HSA–GCSF was generated using these primers, purified, digested with *Bsu* 36I and *Asc* I restriction enzymes, and cloned into the *Bsu* 36I and *Asc* I sites of the pScNHSA vector. After the sequence was confirmed, the expression cassette encoding this GCSF albumin fusion protein was subcloned into pSAC35 as a *Not* I fragment.

[1063] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing confirmed the presence of the expected HSA sequence (see below).

[1064] GCSF albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of GCSF, i.e., Thr-31 to Pro-204. In one embodiment of the invention, GCSF albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature GCSF albumin fusion protein is secreted directly into the culture medium. GCSF albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, GCSF albumin fusion proteins of the invention comprise the native GCSF signal sequence. In further preferred embodiments, the GCSF albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides

encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 1643.

Expression in yeast S. cerevisiae.

[1065] Transformation of construct 1643 into yeast *S. cerevisiae* was carried out by methods known in the art (see Example 3) and as previously described for construct ID 1642 (see Example 19).

Purification from yeast S. cerevisiae cell supernatant.

[1066] A general procedure for purification of albumin fusion proteins is described in Example 4. The cell supernatant containing GCSF albumin fusion protein expressed from construct ID #1643 in yeast *S. cerevisiae* cells was purified according to the following method. Another purification scheme is described in Example 19.

Step 1: Phenyl Sepharose Fast Flow (hs), pH 7.2

[1067] The fermentation supernatant (3.5 L) was adjusted to 139 mS and pH 7.2 with ammonium sulfate to a final concentration of 1 M in 50 mM Tris, pH 7.2. The phenyl sepharose column was loaded at a flow rate of 300 cm/hr. The column was washed with 50 mM Tris-HCl, pH 7.2. A series of lower salt elutions were executed to remove contaminating proteins followed by a WFI elution to elute the target protein. A NaOH strip of the column revealed that a significant portion of the target protein was not removed by previous treatments.

Step 2: Mimetic Blue, pH 6.5

[1068] The eluted target protein was diafiltered with 20 mM citrate phosphate buffer, (CPB), pH 6.5 and then loaded onto a Mimetic Blue column previously equilibrated with 20 mM CPB, pH 6.5 buffer. The column was washed with equilibration buffer for 10 column volumes. The majority of the target protein was then eluted with a 0.2 M NaCl wash. Higher salt concentration elution solutions (1 M and 2 M NaCl) revealed some target protein. However, when HPLC-SEC was performed on these fractions the majority of the target protein was observed as aggregates. This purification step resulted in > 85% purity of the target protein.

Step 3: Q HP, pH 6.5

[1069] The target protein was diluted with 20 mM CPB, pH 6.5 (5-fold) to a conductivity of < 5 mS and loaded onto the Q HP resin. A series of elutions, 100 mM, 200 mM, 500 mM, and 1 M NaCl, were performed. The target protein eluted with 100 mM NaCl.

Step 4: SP FF, pH 5.5

[1070] The target protein was diluted with 20 mM CPB, pH 5.0, and adjusted to pH 5.0. The target protein was loaded onto SP Sepharose FF column. The column was washed with 5 column volumes of equilibration buffer. The 45 kDa contaminating protein, a proteolyzed fragment of HSA, did not bind to the resin and was observed in the load flow thru (LFT). The target protein was eluted in a shallow gradient from 0 – 500 mM NaCl. The target protein eluted at about 250 mM NaCl. The target protein was diafiltered into the final storage buffer of 20 mM CPB, pH 6.5.

[1071] Analysis by SDS-PAGE identified an 88 kDa protein with > 95% purity. N-terminal sequencing resulted in the major sequence being "DAHKS" (SEQ ID NO:2143) which is the amino-terminus of the mature form of HSA. The final buffer composition is 20 mM CPB, pH 6.5. From 3.5 L of culture supernatant, 1.94 mg protein was purified.

The activity of GCSF albumin fusion encoded by construct ID # 1643 can be assayed using an in vitro NFS-60 cell proliferation assay.

Method

[1072] The GCSF albumin fusion protein encoded by construct 1643 was tested in the *in vitro* NFS-60 cell proliferation bioassay previously described in Example 19 under subsection headings, "The activity of GCSF can be assayed using an *in vitro* NFS-60 cell proliferation assay" and "The activity of GCSF albumin fusion encoded by construct ID # 1642 can be assayed using an *in vitro* NFS-60 cell proliferation assay".

Results

[1073] Construct 1643 demonstrated the ability to cause NFS-60 cell proliferation in a dose dependent manner, while the control supernatant with HSA alone did not produce any activity (see Figure 11).

The activity of GCSF albumin fusion encoded by construct ID # 1643 can be assayed in vivo using C57BL/6 mice: GCSF-HSA as a Mobilizing Agent.

Methods

[1074] The GCSF albumin fusion protein encoded by construct 1643 can be assayed

according to the procedure as previously described in Example 19 under subsection headings, "The activity of GCSF can be assayed *in vivo* using C57BL/6 mice: GCSF-HSA as a Mobilizing Agent" and "The activity of GCSF albumin fusion encoded by construct ID # 1642 can be assayed *in vivo* using C57BL/6 mice: GCSF-HSA as a Mobilizing Agent".

EXAMPLE 21: Indications for GCSF albumin fusion proteins.

[1075] Based on the activity of GCSF albumin fusion proteins in the above assays, GCSF albumin fusion proteins are useful in chemoprotection, treating, preventing, and/or diagnosing inflammatory disorders, myelocytic leukemia, primary neutropenias (e.g., Kostmann syndrome), secondary neutropenia, prevention of neutropenia, prevention and treatment of neutropenia in HIV-infected patients, prevention and treatment of neutropenia associated with chemotherapy, infections associated with neutropenias, myelopysplasia, and autoimmune disorders, mobilization of hematopoietic progenitor cells, bone marrow transplant, acute myelogeneous leukemia, non-Hodgkin's lymphoma, acute lymphoblastic leukemia, Hodgkin's disease, accelerated myeloid recovery, and glycogen storage disease.

EXAMPLE 22: Construct ID 2363, GCSF-HSA-EPO.A28-D192, Generation.

[1076] Construct ID 2363, pC4:GCSF.HSA.EPO.A28-D192, comprises DNA encoding a GCSF-HSA–EPO triple fusion protein having the full-length form of the Granulocyte Colony Stimulating Factor, (G-CSF), protein, fused to the amino-terminus of the mature form of HSA, which is fused to the amino-terminus of the mature form of EPO, i.e., amino acids A28-D192, with the exception of the final Arg residue, in the CHO mammalian cell-line expression vector pC4.

Cloning of GCSF-HSA-EPO cDNA

[1077] Construct ID # 1642, i.e., pSAC35:GCSF.T31-P204.HSA (Example 19), was used as a template to generate a part of construct 2363. The following polynucleotides were synthesized:

GCSF/EPO-1: 5'-TGTGGCACAGTGCACTCTGGACAGTGCAGGAAGCCACCC CCCTGGGCCCTGCCAGCTCCC-3' (primer 79388) (SEQ ID NO:1129)

GCSF/EPO-2: 5'-GGCACACTTGAGTCTCTGTTTGGCAGACG-3' (primer 79239) (SEQ ID NO:1130)

GCSF/EPO-3: 5'-ACCCAGAGCCCCATGAAGCTGATGGCCCTGCAGCTGCTGCTG TGGCACAGTGCACTCTGG-3' (primer 79389) (SEQ ID NO:1131)

GCSF/EPO-4: 5'- GGTTG*GGATCC*AAGCTTCCGCCACCATGGCTGGACCTGCCAC CCAGAGCCCCATGAAGCT-3'(primer 79390) (SEQ ID NO:1132)

[1078] The full-length sequence of GCSF was generated in a three-step overlapping PCR reaction using combinations of primers GCSF/EPO-1, GCSF/EPO-2, GCSF/EPO-3, and GCSF/EPO-4. Primers GCSF/EPO-1, GCSF/EPO-3, and GCSF/EPO-4 consist of sequences that span the amino-terminus of the full-length of GCSF. Primer GCSF/EPO-2 comprises of the reverse complement of the sequence that spans amino acids Ser-216 to Ala-225 of HSA. The first PCR reaction included construct 1642 as template and primers GCSF/EPO-1 and GCSF/EPO-2. The product obtained from the first PCR reaction was used as template in the second PCR reaction which included primers GCSF/EPO-3 and GCSF/EPO-2. The product obtained from the second PCR reaction was used as template in the third PCR reaction which included primers GCSF/EPO-4 and GCSF/EPO-2. Primer GCSF/EPO-4 has a *Bam* HI site (shown in italics) followed by the Kozak sequence (shown underlined). The final PCR product contains a 5' *Bam* HI restriction site followed by an appropriate Kozak sequence, the entire full-length GCSF coding sequence and part of the HSA open reading frame from Asp-25 – Ala-225. The *Cla* I site is inherent in the polynucleotide sequence of the mature form of HSA and is localized in close proximity to the 5'-end of the mature form of HSA. The *Bam* HI-*Cla* I fragment was cloned into similarly digested pC4.HSA.EPO.A28-D192 construct ID # 1981.

[1079] Construct ID #2363 encodes an albumin fusion protein containing the leader and mature forms of GCSF, followed by the mature HSA protein, followed by the mature form of EPO.

[1080] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing can confirm the presence of the expected GCSF sequence (see below).

[1081] GCSF/EPO albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of GCSF, i.e., Thr-31 to Pro-204, and fused to either the N- or C-terminus of the mature form of EPO, i.e.,Ala-28 to Asp-192. In one embodiment of the invention, GCSF/EPO albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature GCSF/EPO albumin fusion protein is secreted directly into the culture medium. GCSF/EPO albumin fusion proteins of the invention may comprise

heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, GCSF/EPO albumin fusion proteins of the invention comprise either the native GCSF or the native EPO signal sequence. In further preferred embodiments, the GCSF/EPO albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2363.

Expression in CHO cells.

[1082] Construct 2363 can be transfected into CHO cells as previously described in Examples 6 and 8.

Purification from CHO supernatant.

[1083] A general purification procedure for albumin fusion proteins has been described in Example 7. The triple fusion protein GCSF-HSA-EPO encoded by construct 2363 can be purified as previously described in Examples 7 and 9. N-terminal sequencing should yield the sequence TPLGP (SEQ ID NO:2144) which corresponds to the mature form of GCSF.

The activity of GCSF-HSA-EPO encoded by construct ID # 2363 can be assayed using an in vitro TF-1 cell proliferation assay and an in vitro NFS-60 cell proliferation assay.

Method

[1084] The activity of the triple fusion protein GCSF-HSA-EPO encoded by construct 2363 was assayed in the *in vitro* TF-1 cell proliferation assay as previously described under subsection heading, "*In vitro* TF-1 cell proliferation assay for construct 1981", in Example 9, as well as in the *in vitro* NFS-60 cell proliferation assay as previously described under subsection heading, "The activity of GCSF albumin fusion encoded by construct ID # 1642 can be assayed using an *in vitro* NFS-60 cell proliferation assay", in Example 19.

Result

[1085] THE GCSF-HSA-EPO albumin fusion encoded by construct 2363 demonstrated proliferation of both TF-1 cells and NFS-60 cells.

The activity of GCSF-HSA-EPO albumin fusion encoded by construct ID # 2363 can be assayed in vivo.

[1086] The activity of the triple fusion protein GCSF-HSA-EPO encoded by construct 2363 can be assayed in the *in vivo* Harlan mouse model to measure hematocrit levels as previously described in Example 9 under subsection heading, "The activity of construct 1981 can be assayed using an *in vivo* Harlan mouse model for measuring hematocrit", as well as in C57BL/6 mice where GCSF-HSA-EPO is a mobilizing agent as previously described in Example 19 under subsection heading, "The activity of GCSF albumin fusion encoded by construct ID # 1642 can be assayed *in vivo* using C57BL/6 mice: GCSF-HSA as a Mobilizing Agent".

EXAMPLE 23: Construct ID 2373, GCSF-HSA-EPO.A28-D192, Generation.

[1087] Construct ID 2373, pC4:GCSF.HSA.EPO.A28-D192.R140G, comprises DNA encoding a GCSF-HSA–EPO triple fusion protein which has the full-length form of the Granulocyte Colony Stimulating Factor, "G-CSF", protein, fused to the amino-terminus of the mature form of HSA, which is fused to the amino-terminus of the mature form of EPO, i.e., A28-D192 which has the Arg-140 to Gly mutation, in the CHO mammalian cell-line expression vector pC4.

Cloning of EPO cDNA for construct 2373

[1088] Construct ID #2373 encodes an albumin fusion protein containing the leader sequence and the mature form of GCSF, followed by the mature HSA protein followed by the mature form of EPO which has the Arg-140 to Gly mutation (SEQ ID NO:401). Construct ID #2373 was generated by using construct ID #2363, i.e., pC4:GCSF.HSA.EPO.R140G as a template for PCR mutagenesis.

[1089] Four oligonucleotides suitable for PCR amplification of template of construct ID #2363, GCSF/EPO-5, GCSF/EPO-6, GCSF/EPO-7, and GCSF/EPO-8, were synthesized.

GCSF/EPO-5: 5'-GTTGAAAGTAAGGATGTTTG-3' (primer 78219) (SEQ ID NO:1125)

GCSF/EPO-6: 5'-CCTTCTGGGC**TCC**CAGAGCCCGAAG-3' (primer 82847) (SEQ ID NO:1126)

GCSF/EPO-7: 5'-CTTCGGGCTCTG**GGA**GCCCAGAAGG-3' (primer 82846) (SEQ ID NO:1127)

GCSF/EPO-8:5'-ACCAGGTAGAGAGCTTCCACC -3' (pC3') (SEQ ID NO:1128)

[1090] Construct 2373 was generated by nested PCR amplification using construct 2363 as the template. In the first round of PCR amplifications, the N-terminal and the C-terminal fragments of construct ID 2363 were independently amplified. The N-terminal fragment was generated using primers GCSF/EPO-5 and GCSF/EPO-6. The GCSF/EPO-5 corresponds to the nucleic acid sequence that encodes for amino acid residues 334 to 340 of the full-length form of HSA. The GCSF/EPO-6 primer comprises the reverse complement of the sequence spanning amino acids 136 to 143 of the full-length form of EPO with the exception that the codon CGA encoding the Arg residue at amino acid 140 (highlighted in bold) is altered to the codon GGA which encodes a Gly residue. The C-terminal fragment was generated using primers GCSF/EPO-7 and GCSF/EPO-8. The GCSF/EPO-7 primer comprises the nucleic acid sequence encoding amino acids 136 to 143 of the full-length form of EPO with the exception that the codon CGA encoding the Arg residue at amino acid 140 (highlighted in bold) is altered to the codon GGA which encodes a Gly residue. In GCSF/EPO-8, the sequence comprises nucleotides within the pC4 vector downstream of the stop codon. In the second round of PCR amplifications, primers GCSF/EPO-5 and GCSF/EPO-8 were used to amplify the GCSF-HSA–EPO triple fusion protein which has the full-length form of G-CSF fused to the amino-terminus of the mature form of HSA, which is fused to the amino-terminus of the mature form of EPO, i.e., A28-D192 which has the Arg-140 to Gly mutation. The reaction mixture contained both the PCR amplified N-terminal fragment and the PCR amplified C-terminal fragment.

[1091] The PCR product was purified and then digested with *Bsu36*I and *Asc*I. After further purification of the *Bsu36*I-*Asc*I fragment by gel electrophoresis, the product was cloned into *Bsu36*I/*Asc*I digested construct 2363 to give construct ID # 2373.

[1092] Further, analysis of the N-terminus of the albumin fusion protein by amino acid sequencing can confirm the presence of the expected GCSF sequence (see below).

[1093] GCSF/EPO albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of GCSF, i.e., Thr-31 to Pro-204, and fused to either the N- or C-terminus of the mature form of EPO, i.e.,Ala-28 to Asp-192. In one embodiment of the invention, GCSF/EPO albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature GCSF/EPO albumin fusion protein is secreted directly

into the culture medium. GCSF/EPO albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, GCSF/EPO albumin fusion proteins of the invention comprise either the native GCSF or the native EPO signal sequence. In further preferred embodiments, the GCSF/EPO albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2373.

Expression in CHO cells.

[1094] Construct 2373 can be transfected into CHO cells as previously described in Examples 6 and 8.

Purification from CHO supernatant.

[1095] A general purification procedure for albumin fusion proteins has been described in Example 7. The triple fusion protein GCSF-HSA-EPO.R140G encoded by construct 2373 can be purified as previously described in Examples 7 and 8. N-terminal sequencing should yield the sequence TPLGP (SEQ ID NO:2144) which corresponds to the mature form of GCSF.

The activity of GCSF-HSA-EPO.R140G encoded by construct ID # 2373 can be assayed using an in vitro TF-1 cell proliferation assay and an in vitro NFS-60 cell proliferation assay.

Method

[1096] The activity of the triple fusion protein GCSF-HSA-EPO.R140G encoded by construct 2373 can be assayed in the *in vitro* TF-1 cell proliferation assay as previously described in Example 9 under subsection heading, "*In vitro* TF-1 cell proliferation assay for construct 1981", as well as in the *in vitro* NFS-60 cell proliferation assay as previously described in Example 19 under subsection heading, "The activity of GCSF albumin fusion encoded by construct ID # 1642 can be assayed using an *in vitro* NFS-60 cell proliferation assay".

Result

[1097] THE GCSF-HSA-EPO.R140G albumin fusion encoded by construct 2373 demonstrated proliferation of both TF-1 cells and NFS-60 cells.

The activity of GCSF-HSA-EPO.R140G albumin fusion encoded by construct ID # 2373 can be assayed in vivo.

Method

[1098] The activity of the triple fusion protein GCSF-HSA-EPO.R140G encoded by construct 2373 can be assayed in the *in vivo* Harlan mouse model to measure hematocrit levels as previously described in Example 9 under subsection heading, "The activity of construct 1981 can be assayed using an *in vivo* Harlan mouse model for measuring hematocrit", as well as in C57BL/6 mice where GCSF-HSA-EPO.R140G is a mobilizing agent as previously described in Example 19 under subsection heading, "The activity of GCSF albumin fusion encoded by construct ID # 1642 can be assayed *in vivo* using C57BL/6 mice: GCSF-HSA as a Mobilizing Agent".

EXAMPLE 24: Indications for the GCSF-HSA-EPO Triple Fusion

[1099] Indications for triple fusion proteins comprising GCSF, EPO and HSA, (including, but not limited to, those encoded by constructs 2363 and 2373) may include those indications specified for the EPO albumin fusion proteins and for the GCSF albumin fusion proteins, including but not limited to, bleeding disorders and anemia caused by a variety of conditions, including but not limited to end-stage renal disease (dialysis patients), chronic renal failure in pre-dialysis, zidovudine-treated HIV patients, cancer patients on chemotherapy, and premature infants; pre-surgery in anemic patients undergoing elective non-cardiac, non-vascular surgery to reduce the need for blood transfusions; aplastic and other refractory anemias, refractory anemia in Inflammatory Bowel Disease, and transfusion avoidance in elective orthopedic surgerychemoprotection; treating, preventing, and/or diagnosing inflammatory disorders, myelocytic leukemia, primary neutropenias (e.g., Kostmann syndrome), secondary neutropenia, prevention of neutropenia, prevention and treatment of neutropenia in HIV-infected patients, prevention and treatment of neutropenia associated with chemotherapy, infections associated with neutropenias, myelopysplasia, and autoimmune disorders, mobilization of hematopoietic progenitor cells, bone marrow transplant, acute myelogeneous leukemia, non-Hodgkin's lymphoma, acute lymphoblastic leukemia, Hodgkin's disease, accelerated myeloid recovery, and glycogen storage disease.

EXAMPLE 25: Construct ID 2053, IFNb-HSA, Generation.

[1100] Construct ID 2053, pEE12.1:IFNb.HSA, comprises DNA encoding an IFNb albumin fusion protein which has the full-length IFNb protein including the native IFNb leader sequence fused to the amino-terminus of the mature form of HSA in the NS0 expression vector pEE12.1.

Cloning of IFNb cDNA

[1101] The polynucleotide encoding IFNb was PCR amplified using primers IFNb-1 and IFNb-2, described below, cut with *Bam* HI/*Cla* I, and ligated into *Bam* HI/*Cla* I cut pC4:HSA, resulting in construct 2011. The *Eco* RI/*Eco* RI fragment from Construct ID # 2011 was subcloned into the *Eco* RI site of pEE12.1 generating construct ID #2053 which which comprises DNA encoding an albumin fusion protein containing the leader sequence and the mature form of IFNb, followed by the mature HSA protein.

[1102] Two oligonucleotides suitable for PCR amplification of the polynucleotide encoding the full-length of IFNb, IFNb-1 and IFNb-2, were synthesized:

IFNb-1: 5'- GCGCGGATCCGAATT*CCGCCGCCATG*ACCAACAAGTGTCTCCTCCA AATTGCTCTCCTGTTGTGCTTCTCCACTACAGCTCTTTCCATGAGCTACAACTTGC TTGG-3' (SEQ ID NO:817)

IFNb-2: 5'- GCGCGCATCGATGAGCAACCTCACTCTTGTGTGCATCGTTTCGGA GGTAACCTGT-3' (SEQ ID NO:818)

[1103] The IFNb-1 primer incorporates a *Bam* HI cloning site (shown underlined), an *Eco* RI cloning site, and a Kozak sequence (shown in italics), followed by 80 nucleotides encoding the first 27 amino acids of the full-length form of IFNb. In IFNb-2, the *Cla* I site (shown underlined) and the DNA following it are the reverse complement of DNA encoding the first 10 amino acids of the mature HSA protein (SEQ ID NO:1038) and the last 18 nucleotides are the reverse complement of DNA encoding the last 6 amino acid residues of IFNb (see Example 2). A PCR amplimer was generated using these primers, purified, digested with *Bam* HI and *Cla* I restriction enzymes, and cloned into the *Bam* HI and *Cla* I sites of the pC4:HSA vector. After the sequence was confirmed, an *Eco* RI fragment containing the IFNb albumin fusion protein expression cassette was subcloned into *Eco* RI digested pEE12.1.

[1104] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing can confirm the presence of the expected IFNb sequence (see below).

[1105] IFNb albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of IFNb, i.e., Met-22 to Asn-187. In one embodiment of the invention, IFNb albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature IFNb albumin fusion protein is secreted directly into the culture medium. IFNb albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, IFNb albumin fusion proteins of the invention comprise the native IFNb. In further preferred embodiments, the IFNb albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2053.

Expression in murine myeloma NS0 cell-lines.

[1106] Construct ID # 2053, pEE12.1:IFNb-HSA , was electroporated into NS0 cells by methods known in the art (see Example 6).

Purification from NS0 cell supernatant.

[1107] Purification of IFNb-HSA from NS0 cell supernatant may follow the methods described in Example 10 which involve Q-Sepharose anion exchange chromatography at pH 7.4 using a NaCl gradient from 0 to 1 M in 20 mM Tris-HCl, followed by Poros PI 50 anion exchange chromatography at pH 6.5 with a sodium citrate gradient from 5 to 40 mM, and diafiltrating for 6 DV into 10 mM citrate, pH 6.5 and 140 mM NaCl, the final buffer composition. N-terminal sequencing should yield the sequence MSYNLL which is the amino terminus of the mature form of IFNb. The protein has an approximate MW of 88.5 kDa.

[1108] For larger scale purification, e.g., 50 L of NS0 cell supernatant can be concentrated into ~8 to 10 L. The concentrated sample can then be passed over the Q-Sepharose anion exchange column (10 x 19 cm, 1.5 L) at pH 7.5 using a step elution consisting of 50 mM NaOAc, pH 6.0 and 150 mM NaCl. The eluted sample can then be virally inactivated with 0.75% Triton-X 100 for 60 min at room temperature. SDR-Reverse

Phase chromatography (10 cm x 10 cm, 0.8 L) can then be employed at pH 6.0 with 50 mM NaOAc and 150 mM NaCl, or alternatively, the sample can be passed over an SP-sepharose column at pH 4.8 using a step elution of 50 mM NaOAc, pH 6.0, and 150 mM NaCl. DV 50 filtration would follow to remove any viral content. Phenyl-650M chromatography (20 cm x 12 cm, 3.8 L) at pH 6.0 using a step elution consisting of 350 mM $(NH_4)_2SO_4$ and 50 mM NaOAc, or alternatively consisting of 50 mM NaOAc pH 6.0, can follow. Diafiltration for 6-8 DV will allow for buffer exchange into the desired final formulation buffer of either 10 mM Na_2HPO_4 + 58 mM sucrose + 120 mM NaCl, pH 7.2 or 10 mM citrate, pH 6.5, and 140 mM NaCl or 25 mM Na_2HPO_4, 100 mM NaCl, pH 7.2.

The activity of IFNb can be assayed using an in vitro ISRE-SEAP assay.

[1109] All type I Interferon proteins signal through a common receptor complex and a similar Jak/STAT signaling pathway that culminates in the activation of Interferon, "IFN", responsive genes through the Interferon Sequence Responsive Element, "ISRE". A convenient assay for type I IFN activity is a promoter-reporter based assay system that contains multiple copies of the ISRE element fused to a downstream reporter gene. A stable HEK293 cell-line can be generated and contains a stably integrated copy of an ISRE-SEAP reporter gene that is extremely sensitive to type I IFNs and displays linearity over 5 logs of concentration.

Method of Screening of IFNb-HSA NS0 stable clones.

[1110] Construct 2053 was electroporated into NS0 cells as described in Example 6. The NS0 cells transfected with construct ID # 2053 were screened for activity by testing conditioned growth media in the ISRE-SEAP assay. The ISRE-SEAP/293F reporter cells were plated at 3 x 10^4 cell/well in 96-well, poly-D-lysine coated, plates, one day prior to treatment. Reporter cells were treated with various dilutions (including but not limited to 1:500 and 1:5000) of conditioned supernatant or purified preparations of IFNb albumin fusion protein encoded by construct ID 2053 or rhIFNb as a control. The reporter cells were then incubated for 24 hours prior to removing 40 □L for use in the SEAP Reporter Gene Chemiluminescent Assay (Roche catalog # 1779842). Recombinant human Interferon beta, "rhIFNb" (Biogen), was used as a positive control.

Result

[1111] The purified preparation of NS0 expressed IFNb-HSA had a greater EC50 of 9.3 x 10^{-9} g/mL than rhIFNb (Biogen) which had an EC50 of 1.8 x 10^{-10} g/mL (see Figure 13).

In vivo induction of OAS by an Interferon.

Method

[1112] The OAS enzyme, 2'-5'- OligoAdenylate Synthetase, is activated at the transcriptional level by interferon in response to antiviral infection. The effect of interferon constructs can be measured by obtaining blood samples from treated monkeys and analyzing these samples for transcriptional activation of two OAS mRNA, p41 and p69. A volume of 0.5 mL of whole blood can be obtained from 4 animals per group at 7 different time points, day 0, day 1, day 2, day 4, day 8, day 10, and day 14 per animal. The various groups may include injection of vehicle control, intravenous and/or subcutaneous injection of either 30 □g/kg and/or 300 □g/kg IFN albumin fusion protein on day 1, and subcutaneous injection of 40 □g/kg of Interferon alpha (Schering-Plough) as a positive control on days 1, 3, and 5. The levels of the p41 and the p69 mRNA transcripts can be determined by real-time quantitative PCR (Taqman) using probes specific for p41-OAS and p69-OAS. OAS mRNA levels can be quantitated relative to 18S ribosomal RNA endogenous control.

In vivo induction of OAS by Interferon beta albumin fusion encoded by construct ID 2053.

Method

[1113] The activity of the HSA-IFNb fusion protein encoded by construct 2053 can be assayed in the *in vivo* OAS assay as previously described above under subsection heading, "*In vivo* induction of OAS by an Interferon".

EXAMPLE 26: Indications for IFNb albumin fusion proteins.

[1114] IFN beta albumin fusion proteins (including, but not limited to, those encoded by construct 2053) can be used to treat, prevent, ameliorate and/or detect multiple sclerosis. Other indications include, but are not limited to, melanoma, solid tumors, cancer, bacterial infections, chemoprotection, thrombocytopenia, HIV infections, prostate cancer, cancer, hematological malignancies, hematological disorders, preleukemia, glioma, hepatitis B, hepatitis C, human papillomavirus, pulmonary fibrosis, age-related macular degeneration, brain cancer, glioblastoma multiforme, liver cancer, malignant melanoma, colorectal cancer,

Crohn's disease, neurological disorders, non-small cell lung cancer, rheumatoid arthritis, and ulcerative colitis.

EXAMPLE 27: Construct ID 1941, HSA-PTH84, Generation.

[1115] Construct ID 1941, pC4.HSA.PTH84, encodes for an HSA-PTH84 fusion protein which comprises the full-length of HSA including the native HSA leader sequence, fused to the mature form of the human parathyroid hormone, "PTH84" Ser-1 to Gln-84, cloned into the mammalian expression vector pC4.

Cloning of PTH84 cDNA for construct 1941

[1116] The DNA encoding PTH84 was amplified with primers PTH84-1 and PTH84-2, described below, cut with *Bsu* 36I/*Not* I, and ligated into *Bsu* 36I/*Not* I cut pC4:HSA. Construct ID #1941 encodes an albumin fusion protein containing the full-length form of HSA that includes the native HSA leader sequence, followed by the mature PTH84 protein.

[1117] Two primers suitable for PCR amplification of the polynucleotide encoding the mature form of PTH84, PTH84-1 and PTH84-2, were synthesized.

PTH84-1: 5'- GAGCGCG*CCTTAGG*CTCTGTGAGTGAAATACAGCTTATGCATAAC-3' (SEQ ID NO:787)

PTH84-2: 5'-CGGTGC*GCGGCCGC*TTACTGGGATTTAGCTTTAGTTAATACATTCA CATC-3' (SEQ ID NO:788)

[1118] PTH84-1 incorporates a *Bsu* 36I cloning site (shown in italics) followed by the nucleic acid sequence encoding amino acid residues Ala-Leu-Gly corresponding to the end of the mature form of HSA (the last Leu is absent) in conjunction with amino acid residues Ser-1 to Asn-10 of the mature form of PTH84. In PTH84-2, the *Not* I site is shown in italics and the nucleic acid sequence that follows corresponds to the reverse complement of DNA encoding the last 11 amino acids of the mature PTH84 protein. Using these two primers, the PTH84 protein was PCR amplified.

[1119] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing confirmed the presence of the expected HSA sequence (see below).

[1120] PTH84 albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of PTH84, i.e., Ser-1 to Gln-84. In one embodiment of the invention, PTH84 albumin fusion proteins of the invention further comprise a signal sequence which directs the

nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature PTH84 albumin fusion protein is secreted directly into the culture medium. PTH84 albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, PTH84 albumin fusion proteins of the invention comprise the native PTH84. In further preferred embodiments, the PTH84 albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 1941.

Expression in 293T cells.

[1121] Construct 1941 was transfected into 293T cells cells by methods known in the art (e.g., lipofectamine transfection) and selected with 100 nM methotrexate (see Example 6). Expression levels were examined by immunoblot detection with anti-HSA serum as the primary antibody.

Purification from 293T cell supernatant.

[1122] The 293T cell supernatant containing the secreted HSA-PTH84 fusion protein expressed from construct ID #1941 in 293T cells was purified as described in Example 7. Specifically, initial capture was performed with an anionic HQ-50 resin at pH 7.2 using a sodium phosphate buffer (25 mM Na_2HPO_4 pH 7.2) and 16 column volumes of a salt gradient elution of 0 to 0.5 M NaCl, followed by Hydrophobic Interaction Chromatography, "HIC", with the Phenyl 650 M resin (from Tosohaas) using 36 column volumes of a salt gradient elution of 2.75 to 0 M NaCl at pH 7.2 where the sample had a final conductivity of 180 mS. The sample was concentrated using the HQ Poros 50 resin and a salt step elution of 0.15 M NaCl increments. The final buffer composition consisted of 25 mM Na_2HPO_4 + 150 mM NaCl pH 7.2. N-terminal sequencing generated the amino-terminus sequence (i.e., DAHKS, SEQ ID NO:2143) of the mature form of HSA. A protein of approximate MW of 78 kDa was obtained. A final yield of 0.78 mg protein per litre of 293T cell supernatant was obtained.

In vitro induction of cyclic AMP in SaOS2 cells.

Method

[1123] The biological activity of a PTH84 albumin fusion protein can be measured in an *in vitro* assay in which SaOS-2, an osteosarcoma cell-line, is used. PTH activates adenylate cyclase thereby increasing intracellular cyclic AMP levels.

Induction of cAMP in SaOS-2 cells:

[1124] The SaOS-2 cells are subcultured at a density of 8.0 x 10^4 cells/well 24 hours prior to the start of the experiment. On the day of the experiment, the cells are serum starved for 2 hours and then treated for 10 minutes with positive controls (e.g., forskolin at 5 mg/mL), recombinant PTH, or the PTH albumin fusion proteins. Following treatment, the cells are then rinsed and the intracellular cyclic AMP is extracted with cold ethanol. The ethanol extracts can be lyophilized and stored at –80°C for further use. The amount of cyclic AMP present in the samples is quantitated by ELISA as per the manufacturer's protocol (Amersham Life Sciences, Inc.).

In vitro induction of cyclic AMP in SaOS2 cells by the albumin fusion protein encoded by construct 1941.

Method

[1125] The *in vitro* assay to measure the induction of cyclic AMP in SaOS2 cells by the PTH albumin fusion protein encoded by construct 1941 can be carried out as previously described above.

In vivo: Induced release of calcium in TPTX animals.

Methods

[1126] PTH activity is tested by monitoring the PTH albumin fusion proteins ability to reduce the demineralization of bone following ThyroParaThyroidectomy, "TPTX", administration of a low calcium diet, and parathyroid hormone treatment.

[1127] The animals display a variability in pharmacological response as suggested by Votta, *et al.*, 1997, J. Bone and Mineral Res., 12: 1396 – 1406; Millest, *et al.*, 1997, Bone, 20: 465 – 471; and Iwata, *et al.*, 1997, Arthritis and Rheumatism, 40: 499 – 509. Therefore, between 5 and 8 thyroparathyroidectomized animals (purchased from an outside vendor) per group are used. The animals receive replacement injections of thyroxine every other day.

Each experiment will include several groups: (1) placebo and parathyroid hormone (PTH 1-34) injected groups which correspond to the negative and positive controls, respectively; (2-5) PTH albumin fusion proteins, at various concentrations ranging from 0.1 to 12 μg/kg, injected intravenously, intraperitoneally, subcutaneously, and intramuscular, either before, during, or after parathyroid hormone treatment; (6) for some experiments, a cysteine protease inhibitor is tested.

[1128] Under isofluorane anesthesia, the left femoral vein and either the left femoral artery or carotid artery is cannulated with PE-10 tubing fused to PE-50 polyethylene tubing filled with heparinized saline. The catheters are tunneled subcutaneously, exteriorized at the nape and secured to the skin. The animals are allowed to recover for approximately 18 hours prior to being used for experimentation during which time they are given a calcium free diet. During the course of the experiment, 3 blood samples (200 mL each) are taken via the carotid or femoral catheter following 2, 4, and 6 hours of infusion. Longer time points, e.g., 18 hours, may also be desirable.

[1129] A dose relationship between human PTH 1 – 34, the positive control, and the appearance of ionized calcium levels in whole blood was established (data not shown).

The activity of the albumin fusion protein encoded by construct 1941 can be assayed using TPTX animals.

[1130] The activity of the PTH albumin fusion protein encoded by construct 1941 can be measured using TPTX animals and the *in vivo* assay described above under the heading, "*In vivo*: Induced release of calcium in TPTX animals".

An in vivo ovariectomized female rat model.

Methods

[1131] PTH activity is tested by monitoring the ability to induce bone formation in ovariectomized female Lewis or Sprague Dawley rats.

[1132] Surgery is performed on female Lewis or Sprague Dawley rats 8 – 9 weeks of age and experiments are not initiated until 7 to 10 days after the surgery. Samples from blood, urine, and left tibia are obtained weekly from 9 to 12 animals per group. The various groups can include a sham control injected with saline everyday for four weeks, ovariectomized rats injected with saline everyday for four weeks, and ovariectomized rats injected with rat PTH peptide 1-34 at 10 □g/kg subcutaneously five times per week.

Following the fourth and final week of tissue collection, the tibias are sent to Skeletech for bone densitometry analysis.

[1133] The parameters tested are body weight, bone densitometry on left tibia in 70% ethanol, serum pyridinoline from blood, and urine deoxypyridinoline and alpha helical protein. Urine samples are taken in the morning. Blood is obtained from bleeding the heart and the serum is saved for ELISA analysis. Bone densitometry is conducted on the proximal tibia. The left femur can be cut with bone shears just above the knee. The paw can also be removed by cutting the distal tibia. The skin is slit laterally to allow in ethanol and the remainder of the limb is put in a 50 cc tube filled with 70% ethanol. The tube is stored at room temperature until shipped. The rat tibial specimens are allowed to thaw to room temperature the day of the testing. Excised rat tibiae are subjected to bone mineral density determinations using peripheral quantitative computed tomography (pQCT, XCT-RM, Norland/Stratec). The scan is performed at a proximal tibia site (12% of the total length away from the proximal end). One 0.5 mm slice is taken. Scans are analyzed as a whole (total bone) or using a threshold delineation of external and internal boundaries (cortical bone) or an area that is 45% of the total bone tissue by peeling from the outer edge (cancellous bone). Bone mineral density, area and content are then determined by system software. The differences between sham and ovariectomized animals, at each different time point, are determined by two-tailed t-test using SAS statistical software (SAS Institute, Cory, NC). The student t test is used for statistical comparison of means. P values of less than 0.05 are considered statistically significant.

The activity of the albumin fusion protein encoded by construct 1941 can be assayed using the in vivo ovariectomized female rat model.

[1134] The activity of the PTH albumin fusion protein encoded by construct 1941 can be measured using the *in vivo* assay described above under the heading, "An *in vivo* ovariectomized female rat model".

EXAMPLE 28: Construct ID 1949, PTH84-HSA, Generation.

[1135] Construct ID 1949, pC4.PTH84.S1-Q84.HSA, encodes a PTH84-HSA fusion protein which comprises the MPIF leader sequence, followed by the mature form of PTH84, i.e., Ser-1 to Gln-84, fused to the amino-terminus of the mature form of HSA cloned into the mammalian expression vector pC4.

Cloning of PTH84 cDNA for construct 1949

[1136] The DNA encoding PTH84 was amplified with primers PTH84-3 and PTH84-4, described below, cut with *Bam* HI/*SpeI*, and ligated into *Bam* HI/*Xba*I cut pC4:HSA. Construct ID #1949 encodes an albumin fusion protein comprising the mature PTH84 protein followed by the mature form of HSA.

[1137] Two primers suitable for PCR amplification of the polynucleotide encoding the mature form of PTH84, PTH84-3 and PTH84-4, were synthesized.

PTH84-3: 5'-GAGCGC*GGATCC*GCCATCATGAAGGTCTCCGTGGCTGCCCTCTCC TGCCTCATGCTTGTTACTGCCCTTGGATCTCAGGCC**TCTGTGAGTGAAATACAG CTTATGC**-3' (SEQ ID NO:793)

PTH84-4: 5'- GTCGTC*ACTAGT***CTGGGATTTAGCTTTAGTTAATACATTCAC**-3' (SEQ ID NO:794)

[1138] PTH84-3 incorporates a *Bam* HI cloning site (shown in italics) followed by a nucleic acid sequence that encodes the MPIF signal peptide (shown underlined) and amino acid residues Ser-1 to Met-8 (shown in bold) of the mature form of PTH84. In PTH84-4, the *SpeI* site is shown in italics and the nucleic acid sequence that follows corresponds to the reverse complement of DNA encoding the last 10 amino acids of the mature PTH84 protein (shown in bold). Using these two primers, the PTH84 protein was PCR amplified. The PCR amplimer was purified, digested with *Bam* HI and *SpeI* and ligated into *Bam* HI/*Xba*I cut pC4:HSA.

[1139] There are two additional amino acid residues, i.e., Thr and Ser, between PTH84 and HSA as a result of the introduction of the *SpeI* cloning site into the PTH84-4 primer.

[1140] Further analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing confirmed the presence of the expected PTH84 sequence (see below).

[1141] PTH84 albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of PTH84, i.e., Ser-1 to Gln-84. In one embodiment of the invention, PTH84 albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature PTH84 albumin fusion protein is secreted directly into the culture medium.

PTH84 albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, PTH84 albumin fusion proteins of the invention comprise the native PTH84. In further preferred embodiments, the PTH84 albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 1949.

Expression in 293T cells.

[1142] Construct 1949 was transfected into 293T cells cells by methods known in the art (e.g., lipofectamine transfection) and selected with 100 nM methotrexate (see Example 6). Expression levels were examined by immunoblot detection with anti-HSA serum as the primary antibody.

Purification from 293T cell supernatant.

[1143] The 293T cell supernatant containing the secreted PTH84-HSA fusion protein expressed from construct ID #1949 in 293T cells was purified as described in Example 7. Specifically, initial capture was performed with an anionic HQ-50 resin at pH 7.2 using a sodium phosphate buffer (25 mM Na_2HPO_4 pH 7.2) and 16 column volumes of a salt gradient elution of 0 to 0.5 M NaCl, followed by Hydrophobic Interaction Chromatography, "HIC", with the Phenyl 650 M resin (from Tosohaas) using 36 column volumes of a salt gradient elution of 2.75 to 0 M NaCl at pH 7.2 where the sample had a final conductivity of 180 mS. The sample was concentrated using the HQ Poros 50 resin and a salt step elution of 0.15 M NaCl increments. The final buffer composition consisted of 25 mM Na_2HPO_4 + 150 mM NaCl pH 7.2. N-terminal sequencing generated the amino-terminus sequence (i.e., SVSEI, SEQ ID NO:2145) of the mature form of PTH84. A protein of approximate MW of 78 kDa was obtained. A final yield of 0.32 mg protein per litre of 293T cell supernatant was obtained.

In vitro induction of cyclic AMP in SaOS2 cells by the albumin fusion protein encoded by construct 1949.

Result

[1144] A purified HSA-PTH84 albumin fusion protein derived from 293T cells expressing construct 1949 was tested in the *in vitro* assay described in Example 27 under subsection heading, "*In vitro* induction of cyclic AMP in SaOS2 cells". HSA-PTH84 induced an increase in intracellular cyclic AMP levels.

The activity of the albumin fusion protein encoded by construct 1949 can be assayed using TPTX animals.

[1145] The activity of the PTH albumin fusion protein encoded by construct 1949 can be measured using TPTX animals and the *in vivo* assay described in Example 27 under the subsection heading, "*In vivo*: Induced release of calcium in TPTX animals".

The activity of the albumin fusion protein encoded by construct 1949 can be assayed using the in vivo ovariectomized female rat model.

[1146] The activity of the PTH albumin fusion protein encoded by construct 1949 can be measured using the *in vivo* assay described in Example 27 under the subsection heading, "An *in vivo* ovariectomized female rat model".

EXAMPLE 29: Construct ID 2021, PTH84-HSA, Generation.

[1147] Construct ID 2021, pC4.PTH84.S1-Q84.HSA, encodes for an PTH84-HSA fusion protein which comprises the native HSA leader, followed by the mature form of PTH84, i.e., Ser-1 to Gln-84, fused to the amino-terminus of the mature form of HSA cloned into the mammalian expression vector pC4.

Cloning of PTH84 cDNA for construct 2021

[1148] The DNA encoding PTH84 was amplified with primers PTH84-5 and PTH84-6, described below, cut with *Xho* I/*Cla* I, and ligated into *Xho* I/*Cla* I cut pC4:HSA. Construct ID #2021 encodes an albumin fusion protein containing the mature PTH84 protein followed by the mature form of HSA (see Example 5).

[1149] Two primers suitable for PCR amplification of the polynucleotide encoding the mature form of PTH84, PTH84-5 and PTH84-6, were synthesized.

PTH84-5: 5'- CCGCCG*CTCGAG*GGGTGTGTTTCGTCGA**TCTGTGAGTGAAATAC AGCTTATGCATAAC**-3' (SEQ ID NO:823)

PTH84-6: 5'- AGTCCC*ATCGAT*GAGCAACCTCACTCTTGTGTGCATC**CTGGGATT TAGCTTTAGTTAATACATTCACATC**-3' (SEQ ID NO:824)

[1150] PTH84-5 incorporates a *Xho* I cloning site (shown in italics). The *Xho* I site combined with the nucleic acid sequence that follows (shown underlined) encodes for the last four amino acid residues of the chimeric signal peptide of HSA. The nucleic acid sequence in bold encodes for amino acid residues Ser-1 to Asn-10 of the mature form of PTH84. In PTH84-6, the *Cla* I site is shown in italics and the nucleic acid sequence that follows (shown underlined) corresponds to the reverse complement of DNA encoding the first 10 amino acids of the mature form of HSA. The nucleic acid sequence highlighted in bold in PTH84-6 corresponds to the reverse complement of DNA encoding the last 11 amino acids of the mature form of PTH84. Using these two primers, the PTH84 protein was PCR amplified. The PCR amplimer was purified, digested with *Xho* I and *Cla* I and ligated into *Xho* I/*Cla* I cut pC4:HSA.

[1151] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing can confirm the presence of the expected PTH84 sequence (see below).

[1152] PTH84 albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of PTH84, i.e., Ser-1 to Gln-84. In one embodiment of the invention, PTH84 albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature PTH84 albumin fusion protein is secreted directly into the culture medium. PTH84 albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, PTH84 albumin fusion proteins of the invention comprise the native PTH84. In further preferred embodiments, the PTH84 albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2021.

Expression in 293T cells.

[1153] Construct 2021 can be transfected into 293T cells cells by methods known in the art (e.g., lipofectamine transfection) and selected with 100 nM methotrexate (see Example 6). Expression levels can be examined by immunoblot detection with anti-HSA serum as the primary antibody.

Purification from 293T cell supernatant.

[1154] The 293T cell supernatant containing the secreted PTH84-HSA fusion protein expressed from construct ID #2021 in 293T cells can be purified as described in Example 7. Specifically, initial capture can be performed with an anionic HQ-50 resin at pH 7.2 using a sodium phosphate buffer (25 mM Na_2HPO_4 pH 7.2) and 16 column volumes of a salt gradient elution of 0 to 0.5 M NaCl, followed by Hydrophobic Interaction Chromatography, "HIC", with the Phenyl 650 M resin (from Tosohaas) using 36 column volumes of a salt gradient elution of 2.75 to 0 M NaCl at pH 7.2 where the sample has a final conductivity of 180 mS. The sample can be concentrated using the HQ Poros 50 resin and a salt step elution of 0.15 M NaCl increments. The final buffer composition may consist of 25 mM Na_2HPO_4 + 150 mM NaCl pH 7.2. N-terminal sequencing should generate the amino-terminus sequence (i.e., SVSEI) of the mature form of PTH84. A protein of approximate MW of 78 kDa should be obtained.

In vitro induction of cyclic AMP in SaOS2 cells by the albumin fusion protein encoded by construct 2021.

[1155] HSA-PTH84 albumin fusion protein derived from 293T cells expressing construct 2021 can be tested in the *in vitro* assay described in Example 27 under subsection heading, "*In vitro* induction of cyclic AMP in SaOS2 cells".

The activity of the albumin fusion protein encoded by construct 2021 can be assayed using TPTX animals.

[1156] The activity of the PTH albumin fusion protein encoded by construct 2021 can be measured using TPTX animals and the *in vivo* assay described in Example 27 under the subsection heading, "*In vivo*: Induced release of calcium in TPTX animals".

The activity of the albumin fusion protein encoded by construct 2021 can be assayed using

the in vivo ovariectomized female rat model.

[1157] The activity of the PTH albumin fusion protein encoded by construct 2021 can be measured using the *in vivo* assay described in Example 27 under the subsection heading, "An *in vivo* ovariectomized female rat model".

EXAMPLE 30: Indications for PTH84 Albumin Fusion Proteins.

[1158] Results from *in vitro* and *in vivo* assays described above indicate that PTH84 albumin fusion proteins are useful for the treatment, prevention, and/or diagnosis of osteoporosis, malignant hypercalcaemia, and Paget's disease.

EXAMPLE 31: Construct ID 2249, IFNa2-HSA, Generation.

[1159] Construct ID 2249, pSAC35:IFNa2.HSA, comprises DNA encoding an IFNa2 albumin fusion protein which has the HSA chimeric leader sequence, followed by the mature form of IFNa2 protein, i.e., C1-E165, fused to the amino-terminus of the mature form of HSA in the yeast *S. cerevisiae* expression vector pSAC35.

Cloning of IFNa2 cDNA

[1160] The polynucleotide encoding IFNa2 was PCR amplified using primers IFNa2-1 and IFNa2-2, described below. The PCR amplimer was cut with *Sal I/Cla* I, and ligated into *Xho I/Cla* I cut pScCHSA. Construct ID #2249 encodes an albumin fusion protein containing the chimeric leader sequence of HSA, the mature form of IFNa2, followed by the mature HSA protein.

[1161] Two oligonucleotides suitable for PCR amplification of the polynucleotide encoding the mature form of IFNa2, IFNa2-1 and IFNa2-2, were synthesized:

IFNa2-1: 5'-CGCGCGCGTCGACAAAAGA**TGTGATCTGCCTCAAACCCACA**-3' (SEQ ID NO:887)

IFNa2-2: 5'-GCGCGCATCGATGAGCAACCTCACTCTTGTGTGCATC**TTCCTTAC TTCTTAAACTTTCT**-3' (SEQ ID NO:888)

[1162] The IFNa2-1 primer incorporates a *Sal* I cloning site (shown underlined), nucleotides encoding the last three amino acid residues of the chimeric HSA leader sequence, as well as 22 nucleotides (shown in bold) encoding the first 7 amino acid residues of the mature form of IFNa2. In IFNa2-2, the *Cla* I site (shown underlined) and the DNA following

it are the reverse complement of DNA encoding the first 10 amino acids of the mature HSA protein and the last 22 nucleotides (shown in bold) are the reverse complement of DNA encoding the last 7 amino acid residues of IFNa2 (see Example 2). A PCR amplimer of IFNa2-HSA was generated using these primers, purified, digested with *Sal* I and *Cla* I restriction enzymes, and cloned into the *Xho* I and *Cla* I sites of the pScCHSA vector. After the sequence was confirmed, the expression cassette encoding this IFNa2 albumin fusion protein was subcloned into *Not* I digested pSAC35.

[1163] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing can confirm the presence of the expected IFNa2 sequence (see below).

[1164] Other IFNa2 albumin fusion proteins using different leader sequences have been constructed by methods known in the art (see Example 2). Examples of the various leader sequences include, but are not limited to, invertase "INV" (constructs 2343 and 2410) and mating alpha factor "MAF" (construct 2366). These IFNa2 albumin fusion proteins can be subcloned into mammalian expression vectors such as pC4 (constructs 2382) and pEE12.1 as described previously (see Example 5). IFNa2 albumin fusion proteins with the therapeutic portion fused C-terminus to HSA can also be constructed (construct 2381).

[1165] IFNa2 albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of IFNa2, i.e., Cys-1 to Glu-165. In one embodiment of the invention, IFNa2 albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature IFNa2 albumin fusion protein is secreted directly into the culture medium. IFNa2 albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, IFNa2 albumin fusion proteins of the invention comprise the native IFNa2. In further preferred embodiments, the IFNa2 albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2249.

Expression in yeast S. cerevisiae.

[1166] Transformation of construct 2249 into yeast *S. cerevisiae* strain BXP10 was carried out by methods known in the art (see Example 3). Cells can be collected at stationary phase after 72 hours of growth. Supernatants are collected by clarifying cells at 3000g for 10 min. Expression levels are examined by immunoblot detection with anti-HSA serum (Kent Laboratories) or as the primary antibody. The IFNa2 albumin fusion protein of approximate molecular weight of 88.5 kDa can be obtained.

Purification from yeast S. cerevisiae cell supernatant.

[1167] The cell supernatant containing IFNa2 albumin fusion protein expressed from construct ID #2249 in yeast *S. cerevisiae* cells can be purified either small scale over a Dyax peptide affinity column (see Example 4) or large scale by following 5 steps: diafiltration, anion exchange chromatography using DEAE-Sepharose Fast Flow column, hydrophobic interaction chromatography (HIC) using Butyl 650S column, cation exchange chromatography using an SP-Sepharose Fast Flow column or a Blue-Sepharose chromatography, and high performance chromatography using Q-sepharose high performance column chromatography (see Example 4). The IFNa2 albumin fusion protein may elute from the DEAE-Sepharose Fast Flow column with 100 – 250 mM NaCl, from the SP-Sepharose Fast Flow column with 150 – 250 mM NaCl, and from the Q-Sepharose High Performance column at 5 – 7.5 mS/cm. N-terminal sequencing should yield the sequence CDLPQ (SEQ ID NO:2146) which corresponds to the mature form of IFNa2.

The activity of IFNa2 can be assayed using an in vitro ISRE-SEAP assay.

Method

[1168] The IFNa2 albumin fusion protein encoded by construct ID # 2249 can be tested for activity in the ISRE-SEAP assay as previously described in Example 25. Briefly, conditioned yeast supernatants were tested at a 1:1000 dilution for their ability to direct ISRE signal transduction on the ISRE-SEAP/293F reporter cell-line. The ISRE-SEAP/293F reporter cells were plated at 3 x 10^4 cell/well in 96-well, poly-D-lysine coated, plates, one day prior to treatment. The reporter cells were then incubated for 18 or 24 hours prior to removing 40 µL for use in the SEAP Reporter Gene Chemiluminescent Assay (Roche catalog # 1779842). Recombinant human Interferon beta, "rhIFNb" (Biogen), was used as a positive control.

Result

[1169] The purified preparation of IFNa2-HSA demonstrated a relatively linear increase in the ISRE-SEAP assay over concentrations ranging from 10^{-1} to 10^{1} ng/mL (see Figure 15) or 10^{-10} to 10^{-8} ng/mL (see Figure 16).

In vivo induction of OAS by Interferon alpha fusion encoded by construct ID 2249.

Method

[1170] The OAS enzyme, 2'-5'- OligoAdenylate Synthetase, is activated at the transcriptional level by interferon in response to antiviral infection. The effect of interferon constructs can be measured by obtaining blood samples from treated monkeys and analyzing these samples for transcriptional activation of two OAS mRNA, p41 and p69. A volume of 0.5 mL of whole blood was obtained from 4 animals per group at 7 different time points, day 0, day 1, day 2, day 4, day 8, day 10, and day 14 per animal. The various groups include vehicle control, intravenous injection of 30 μg/kg HSA-IFN on day 1, subcutaneous injection of 30 μg/kg of HSA-IFN on day 1, subcutaneous injection of 300 μg/kg of HSA-IFN on day 1, and subcutaneous injection of 40 μg/kg of Interferon alpha (Schering-Plough) as a positive control on days 1, 3, and 5. The levels of the p41 and the p69 mRNA transcripts were determined by real-time quantitative PCR (Taqman) using probes specific for p41-OAS and p69-OAS. OAS mRNA levels were quantitated relative to 18S ribosomal RNA endogenous control. The albumin fusion encoded by construct 2249 can be subjected to similar experimentation.

Results

[1171] A significant increase in mRNA transcript levels for both p41 and p69 OAS was observed in HSA-interferon treated monkeys in contrast to IFNa treated monkeys (see Figure 17 for p41 data). The effect lasted nearly 10 days.

EXAMPLE 32: Indications for IFNa2 Albumin Fusion Proteins

[1172] IFN alpha albumin fusion protein (including, but not limited to, those encoded by constructs 2249, 2343, 2410, 2366, 2382, and 2381) can be used to treat, prevent, ameliorate, and/or detect multiple sclerosis. Other indications include, but are not limited to, Hepatitis C, oncology uses, cancer, hepatitis, human papilloma virus, fibromyalgia, Sjogren's syndrome, hairy cell leukemia, chronic myelogeonus leukemia, AIDS-related Kaposi's sarcoma, chronic hepatitis B, malignant melanoma, non-Hodgkin's lymphoma, external condylomata acuminata, HIV infection, small cell lung cancer, hematological malignancies,

herpes simplex virus infections, multiple sclerosis, viral hemorrhagic fevers, solid tumors, renal cancer, bone marrow disorders, bone disorders, bladder cancer, gastric cancer, hepatitis D, multiple myeloma, type I diabetes mellitus, viral infections, cutaneous T-cell lymphoma, cervical dysplasia, chronic fatigue syndrome, and renal cancer.

[1173] Preferably, the IFNα-albumin fusion protein or IFN hybrid fusion protein is administered in combination with a CCR5 antagonist, further in association with at least one of ribavirin, IL-2, IL-12, pentafuside alone or in combination with an anti-HIV drug therapy, e.g., HAART, for preparation of a medicament for the treatment of HIV-1 infections, HCV, or HIV-1 and HCV co-infections in treatment-naïve as well as treatment-experienced adult and pediatric patients.

EXAMPLE 33: Construct ID 2250, HSA-Insulin (GYG), Generation.

[1174] Construct ID 2250, pSAC35.HSA.INSULIN(GYG).F1-N62, encodes for an HSA-INSULIN (GYG) fusion protein which comprises full length HSA, including the native HSA leader sequence, fused to the amino-terminus of the synthetic single-chain long-acting insulin analog (INSULIN ($GY^{32}G$)) with a Tyr at position 32, cloned into the yeast *S. cerevisiae* expression vector pSAC35.

Cloning of INSULIN (GYG) cDNA for construct 2250.

[1175] The DNA encoding the synthetic single-chain form of INSULIN (GYG) was PCR generated using four overlapping primers. The sequence corresponding to the C-peptide in the middle region of the proinsulin cDNA was replaced by the C-domain of Insulin Growth Factor 1, "IGF-1" (GY^{32}GSSSRRAPQT, SEQ ID NO:2147), to avoid the need for proinsulin processing and to ensure proper folding of the single-chain protein. The sequence was codon optimized for expression in yeast *S. cerevisiae*. The PCR fragment was digested and subcloned into *Bsu* 361/*Asc* I digested pScNHSA. A *Not* I fragment was then subcloned into the pSAC35 plasmid. Construct ID #2250 encodes for full length HSA, including the native HSA leader sequence, fused to the amino-terminus of the synthetic single-chain form of INSULIN (GYG).

[1176] The 5' and 3' primers of the four overlapping oligonucleotides suitable for PCR amplification of the polynucleotide encoding the synthetic single-chain form of INSULIN (GYG), INSULIN (GYG)-1 and INSULIN (GYG)-2, were synthesized:

INSULIN (GYG)-1: 5'-GTCAAGCTG*CCTTAGG*CTTA**TTCGTTAACCAACACTTGTGTGGTT CTCACTTGGTTGAAGCTTTGTACTTGGTTTGTGGTGAA**-3' (SEQ ID NO:889)

INSULIN (GYG)-2: 5'-ATCGCATAT*GGCGCGCC*CTA**TTAGTTACAGTAGTTTTCCAATTG GTACAAAGAACAAATAGAAGTACAA** –3' (SEQ ID NO:890)

[1177] INSULIN (GYG)-1 incorporates a *Bsu* 36I cloning site (shown in italics) and encodes the first 21 amino acids (shown in bold) of the ORF of the synthetic single-chain form of INSULIN (GYG). In INSULIN (GYG)-2, the italicized sequence is an *Asc* I site. In INSULIN (GYG)-2, the bolded sequence is the reverse complement of the last 49 nucleotides encoding amino acid residues Cys-49 to Asn-63 of the synthetic single-chain form of INSULIN (GYG). With these two primers, the synthetic single-chain form of INSULIN (GYG) was PCR amplified. Annealing and extension temperatures and times must be empirically determined for each specific primer pair and template.

[1178] The PCR product was purified (for example, using Wizard PCR Preps DNA Purification System (Promega Corp)) and then digested with *Bsu36*I and *AscI*. After further purification of the *Bsu36*I-*AscI* fragment by gel electrophoresis, the product was cloned into *Bsu36I*/*AscI* digested pScNHSA. A *Not* I fragment was further subcloned into pSAC35 to give construct ID # 2250.

[1179] Further analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing should confirm the presence of the expected mature HSA sequence (see below).

[1180] INSULIN albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the synthetic single-chain analog of INSULIN, i.e., Phe-1 to Asn–62; the sequence corresponding to the C-peptide in the middle region of the proinsulin cDNA was replaced by the C-domain of Insulin Growth Factor 1, "IGF-1" (GY^{32}GSSSRRAPQT, SEQ ID NO:2147). In one embodiment of the invention, INSULIN albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature INSULIN albumin fusion protein is secreted directly into the culture medium. INSULIN albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, INSULIN albumin fusion proteins of the invention comprise the native INSULIN. In further

preferred embodiments, the INSULIN albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2250.

Expression in yeast S. cerevisiae.

[1181] Construct 2250 can be transformed into yeast *S. cerevisiae* by methods known in the art (see Example 3). Expression levels can be examined by immunoblot detection with anti-HSA serum as the primary antibody.

Purification from yeast S. cerevisiae cell supernatant.

[1182] The cell supernatant containing the secreted INSULIN (GYG) albumin fusion protein expressed from construct ID #2250 in yeast *S. cerevisiae* can be purified as described in Example 4. N-terminal sequencing of the albumin fusion protein should result in the sequence DAHKS (SEQ ID NO:2143) which corresponds to the amino terminus of the mature form of HSA.

In vitro [^{3}H]-2-Deoxyglucose Uptake Assay in the presence of the albumin fusion protein encoded by construct 2250.

Method

[1183] The *in vitro* assay to measure the glucose uptake in 3T3-L1 adipocytes in the presence of the INSULIN (GYG) albumin fusion protein encoded by construct 2250 was carried out as described below in Example 41. Other assays known in the art that may be used to test INSULIN (GYG) albumin fusion proteins' include, but are not limited to, L6 Rat Myoblast Proliferation Assay via glycogen synthase kinase-3 (GSK-3) and H4IIe reporter assays (see Example 48) including the rat Malic Enzyme Promoter (rMEP)-SEAP, Sterol Regulatory Element Binding Protein (SREBP)-SEAP, Fatty Acid Synthetase (FAS)-SEAP, and PhosphoEnolPyruvate CarboxyKinase (PEPCK)-SEAP reporters.

Result

[1184] The supernatant derived from transformed yeast *S. cerevisiae* expressing insulin albumin fusion encoded by construct 2250 demonstrated glucose uptake/transport activity in 3T3-L1 adipocytes (see Figure 18).

In vitro Pancreatic Cell-lines Proliferation Assay in the presence of the albumin fusion

protein encoded by construct 2250.

Method

[1185] The *in vitro* assay to measure the differentiation and proliferation of ductal epithelium pancreatic ARIP cell-line into insulin-producing beta cells and/or to measure the proliferation of the insulin-producing RIN-M beta cell-line in the presence of the INSULIN (GYG) albumin fusion protein encoded by construct 2250 can be carried out as described below under heading: "Example 42: *In vitro* Assay of [^{3}H]-Thymidine Incorporation into Pancreatic Cell-lines".

The activity of the albumin fusion protein encoded by construct 2250 can be assayed in vivo using diabetic NOD and/or NIDDM mouse models.

[1186] The activity of the INSULIN (GYG) albumin fusion protein encoded by construct 2250 can be measured using NOD and/or NIDDM mouse models described below under the headings, "Example 44: Occurrence of Diabetes in NOD Mice", "Example 45: Histological Examination of NOD Mice", and "Example 47: *In vivo* Mouse Model of NIDDM".

EXAMPLE 34: Construct ID 2255, Insulin (GYG)-HSA, Generation.

[1187] Construct ID 2255, pSAC35.INSULIN(GYG).F1-N62.HSA, encodes for an INSULIN (GYG)-HSA fusion protein which comprises the HSA chimeric leader sequence of HSA fused to the amino-terminus of the synthetic single-chain long-acting insulin analog (INSULIN (GY^{32}G)) with a Tyr in position 32, which is, in turn, fused to the mature form of HSA, cloned into the yeast *S. cerevisiae* expression vector pSAC35.

Cloning of INSULIN (GYG) cDNA for construct 2255.

[1188] The DNA encoding the synthetic single-chain form of INSULIN (GYG) was PCR generated using four overlapping primers. The sequence corresponding to the C-peptide in the middle region of the proinsulin cDNA was replaced by the C-domain of Insulin Growth Factor 1, "IGF-1" (GY^{32}GSSSRRAPQT, SEQ ID NO:2147), to avoid the need for proinsulin processing and to ensure proper folding of the single-chain protein. The sequence was codon optimized for expression in yeast *S. cerevisiae*. The PCR fragment was digested with *Sal I/Cla* I and subcloned into *Xho I/Cla* I digested pScCHSA. A *Not* I fragment was then subcloned into the pSAC35 plasmid. Construct ID #2255 encodes for the chimeric

leader sequence of HSA fused to the amino-terminus of the synthetic single-chain form of INSULIN (GYG) followed by the mature form of HSA.

[1189] The 5' and 3' primers of the four overlapping oligonucleotides suitable for PCR amplification of the polynucleotide encoding the synthetic single-chain form of INSULIN (GYG), INSULIN (GYG)-3 and INSULIN (GYG)-4, were synthesized:

INSULIN (GYG)-3: 5'-TCCAGGAGC*GTCGAC*AAAAGA**TTCGTTAACCAACACTTGTGTGG TTCTCACTTGGTTGAAGCTTTGTACTTGGTTTGTGGTGAA** -3' (SEQ ID NO:895)

INSULIN (GYG)-4: 5'-AGACTTTAA*ATCGAT*GAGCAACCTCACTCTTGTGTGCATC**GTTAC AGTAGTTTTCCAATTGGTACAAAGAACAAATAGAAGTACAA**–3' (SEQ ID NO:896)

INSULIN (GYG)-3 incorporates a *Sal* I cloning site (shown in italics) and the DNA encoding the first 21 amino acids (shown in bold) of the ORF of the synthetic single-chain form of INSULIN(GYG). In INSULIN (GYG)-4, the italicized sequence is a *Cla* I site; and the *Cla* I site and the DNA following it are the reverse complement of DNA encoding the first 10 amino acids of the mature HSA protein. The bolded sequence is the reverse complement of the 46 nucleotides encoding the last 15 amino acid residues Cys-49 to Asn-63 of the synthetic single-chain form of INSULIN (GYG). With these two primers, the synthetic single-chain INSULIN (GYG) protein was generated by annealing, extension of the annealed primers, digestion with *Sal* I and *Cla* I, and subcloning into *Xho* I/*Cla* I digested pScCHSA. The *Not* I fragment from this clone was then ligated into the *Not* I site of pSAC35 to generate construct ID 2255. Construct ID #2255 encodes an albumin fusion protein containing the chimeric leader sequence, the synthetic single-chain form of INSULIN (GYG), and the mature form of HSA.

[1190] Further analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing should confirm the presence of the expected INSULIN (GYG) sequence (see below).

[1191] INSULIN albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the synthetic single-chain analog of INSULIN, i.e., Phe-1 to Asn–62; the sequence corresponding to the C-peptide in the middle region of the proinsulin cDNA was replaced by the C-domain of Insulin Growth Factor 1, "IGF-1" (GY^{32}GSSSRRAPQT, SEQ ID NO:2147). In one embodiment of the invention, INSULIN albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the

signal peptide encoded by the signal sequence is removed, and the mature INSULIN albumin fusion protein is secreted directly into the culture medium. INSULIN albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, INSULIN albumin fusion proteins of the invention comprise the native INSULIN. In further preferred embodiments, the INSULIN albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2255.

Expression in yeast S. cerevisiae.

[1192] Construct 2255 can be transformed into yeast *S. cerevisiae* by methods known in the art (see Example 3). Expression levels can be examined by immunoblot detection with anti-HSA serum as the primary antibody.

Purification from yeast S. cerevisiae cell supernatant.

[1193] The cell supernatant containing the secreted INSULIN (GYG) albumin fusion protein expressed from construct ID #2255 in yeast *S. cerevisiae* can be purified as described in Example 4. N-terminal sequencing of the expressed and purified albumin fusion protein should generate FVNQH which corresponds to the amino terminus of the synthetic single-chain long-acting insulin analog (INSULIN ($GY^{32}G$)).

In vitro [^{3}H]-2-Deoxyglucose Uptake Assay in the presence of the albumin fusion protein encoded by construct 2255.

Method

[1194] The *in vitro* assay to measure the glucose uptake in 3T3-L1 adipocytes in the presence of the INSULIN (GYG) albumin fusion protein encoded by construct 2255 can be carried out as described below in Example 41. Other assays known in the art that may be used to test INSULIN (GYG) albumin fusion proteins' include, but are not limited to, L6 Rat Myoblast Proliferation Assay via glycogen synthase kinase-3 (GSK-3) and H4IIe reporter assays (see Example 48) including the rat Malic Enzyme Promoter (rMEP)-SEAP, Sterol Regulatory Element Binding Protein (SREBP)-SEAP, Fatty Acid Synthetase (FAS)-SEAP,

and PhosphoEnolPyruvate CarboxyKinase (PEPCK)-SEAP reporters.

In vitro Pancreatic Cell-lines Proliferation Assay in the presence of the albumin fusion protein encoded by construct 2255.

Method

[1195] The *in vitro* assay to measure the differentiation and proliferation of ductal epithelium pancreatic ARIP cell-line into insulin-producing beta cells and/or to measure the proliferation of the insulin-producing RIN-M beta cell-line in the presence of the INSULIN (GYG) albumin fusion protein encoded by construct 2255 can be carried out as described below under heading: "Example 42: *In vitro* Assay of [^{3}H]-Thymidine Incorporation into Pancreatic Cell-lines".

The activity of the albumin fusion protein encoded by construct 2255 can be assayed in vivo using diabetic NOD and/or NIDDM mouse models.

[1196] The activity of the INSULIN (GYG) albumin fusion protein encoded by construct 2255 can be measured using NOD and/or NIDDM mouse models described below under the headings, "Example 44: Occurrence of Diabetes in NOD Mice", "Example 45: Histological Examination of NOD Mice", and "Example 47: *In vivo* Mouse Model of NIDDM".

EXAMPLE 35: Construct ID 2276, HSA-Insulin (GGG), Generation.

[1197] Construct ID 2276, pSAC35.HSA.INSULIN(GGG).F1-N58, encodes for an HSA-INSULIN (GGG) fusion protein which comprises full length HSA, including the native HSA leader sequence fused to the amino-terminus of the synthetic single-chain long-acting insulin analog (INSULIN (GG^{32}G)) with a Gly at position 32, cloned into the yeast *S. cerevisiae* expression vector pSAC35.

Cloning of INSULIN (GGG) cDNA for construct 2276.

[1198] The DNA encoding the synthetic single-chain form of INSULIN (GGG) was PCR generated using four overlapping primers. The sequence corresponding to the C-peptide in the middle region of the proinsulin cDNA was replaced by the synthetic linker "GG^{32}GPGKR" (SEQ ID NO:2148) to avoid the need for proinsulin processing and to ensure proper folding of the single-chain protein. The sequence was codon optimized for expression

in yeast *S. cerevisiae*. The PCR fragment was digested and subcloned into *Bsu* 361/*Asc* I digested pScNHSA. A *Not* I fragment was then subcloned into the pSAC35 plasmid. Construct ID #2276 encodes for full length HSA, including the native HSA leader sequence fused to the amino-terminus of the synthetic single-chain form of INSULIN (GGG).

[1199] The 5' and 3' primers of the four overlapping oligonucleotides suitable for PCR amplification of the polynucleotide encoding the synthetic single-chain form of INSULIN (GGG), INSULIN (GGG)-1 and INSULIN (GGG)-2, were synthesized:

INSULIN (GGG)-5: 5'-GTCAAGCTG*CCTTAGG*CTTA**TTCGTTAACCAACACTTGTGTGGTT CTCACTTGGTTGAAGCTTTGTACTTGGTTTGTGGTGAA**-3' (SEQ ID NO:901)

INSULIN (GGG)-6: 5'-ATCGCATAT*GGCGCGCCC*TA**TTAGTTACAGTAGTTTTCCAATTG GTACAAAGAACAAATAGAAGTACAA** –3' (SEQ ID NO:902)

[1200] INSULIN (GGG)-5 incorporates a *Bsu* 36I cloning site (shown in italics) and encodes the first 21 amino acids (shown in bold) of the ORF of the synthetic single-chain form of INSULIN (GGG). In INSULIN (GGG)-6, the italicized sequence is an *Asc* I site. In INSULIN (GGG)-6, the bolded sequence is the reverse complement of the last 49 nucleotides encoding amino acid residues Cys-44 to Asn-58 of the synthetic single-chain form of INSULIN (GGG). With these two primers, the synthetic single-chain form of INSULIN (GGG) was PCR amplified. Annealing and extension temperatures and times must be empirically determined for each specific primer pair and template.

[1201] The PCR product was purified (for example, using Wizard PCR Preps DNA Purification System (Promega Corp)) and then digested with *Bsu36*I and *AscI*. After further purification of the *Bsu36*I-*AscI* fragment by gel electrophoresis, the product was cloned into *Bsu36*I/*AscI* digested pScNHSA. A *Not* I fragment was further subcloned into pSAC35 to give construct ID # 2276.

[1202] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing should confirm the presence of the expected mature HSA sequence (see below).

[1203] INSULIN albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the synthetic single-chain analog of INSULIN, i.e., Phe-1 to Asn–58; the sequence corresponding to the C-peptide in the middle region of the proinsulin cDNA was replaced by the synthetic linker "GG^{32}GPGKR" (SEQ ID NO:2148). In one embodiment of the invention, INSULIN albumin fusion proteins of the invention further comprise a signal

sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature INSULIN albumin fusion protein is secreted directly into the culture medium. INSULIN albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, INSULIN albumin fusion proteins of the invention comprise the native INSULIN. In further preferred embodiments, the INSULIN albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2276.

Expression in yeast S. cerevisiae.

[1204] Construct 2276 can be transformed into yeast *S. cerevisiae* by methods known in the art (see Example 3). Expression levels can be examined by immunoblot detection with anti-HSA serum as the primary antibody.

Purification from yeast S. cerevisiae cell supernatant.

[1205] The cell supernatant containing the secreted INSULIN (GGG) albumin fusion protein expressed from construct ID #2276 in yeast *S. cerevisiae* can be purified as described in Example 4. N-terminal sequencing should yield DAHKS (SEQ ID NO:2143) which corresponds to the amino terminus of the mature form of HSA.

In vitro [^{3}H]-2-Deoxyglucose Uptake Assay in the presence of the albumin fusion protein encoded by construct 2276.

Method

[1206] The *in vitro* assay to measure the glucose uptake in 3T3-L1 adipocytes in the presence of the INSULIN (GGG) albumin fusion protein encoded by construct 2276 was carried out as described below in Example 41. Other assays known in the art that may be used to test INSULIN (GGG) albumin fusion proteins' include, but are not limited to, L6 Rat Myoblast Proliferation Assay via glycogen synthase kinase-3 (GSK-3) and H4IIe reporter assays (see Example 48) including the rat Malic Enzyme Promoter (rMEP)-SEAP, Sterol

Regulatory Element Binding Protein (SREBP)-SEAP, Fatty Acid Synthetase (FAS)-SEAP, and PhosphoEnolPyruvate CarboxyKinase (PEPCK)-SEAP reporters.

Result

[1207] The supernatant derived from transformed yeast *S. cerevisiae* expressing insulin albumin fusion encoded by construct 2276 demonstrated glucose uptake/transport activity in 3T3-L1 adipocytes (see Figure 18).

In vitro Pancreatic Cell-lines Proliferation Assay in the presence of the albumin fusion protein encoded by construct 2276.

Method

[1208] The *in vitro* assay to measure the differentiation and proliferation of ductal epithelium pancreatic ARIP cell-line into insulin-producing beta cells and/or to measure the proliferation of the insulin-producing RIN-M beta cell-line in the presence of the INSULIN (GGG) albumin fusion protein encoded by construct 2276 can be carried out as described below under heading: "Example 42: *In vitro* Assay of [^{3}H]-Thymidine Incorporation into Pancreatic Cell-lines".

The activity of the albumin fusion protein encoded by construct 2276 can be assayed in vivo using diabetic NOD and/or NIDDM mouse models.

[1209] The activity of the INSULIN (GGG) albumin fusion protein encoded by construct 2276 can be measured using NOD and/or NIDDM mouse models described below under the headings, "Example 44: Occurrence of Diabetes in NOD Mice", "Example 45: Histological Examination of NOD Mice", and "Example 47: *In vivo* Mouse Model of NIDDM".

EXAMPLE 36: Construct ID 2278, Insulin (GGG)-HSA, Generation.

[1210] Construct ID 2278, pSAC35.INSULIN(GGG).HSA, encodes for an INSULIN (GGG)-HSA fusion protein which comprises the HSA chimeric leader sequence of HSA fused to the amino-terminus of the synthetic single-chain long-acting insulin analog (INSULIN (GG^{32}G)) with a Gly in position 32, which is, in turn, fused to the mature form of HSA, cloned into the yeast *S. cerevisiae* expression vector pSAC35.

Cloning of INSULIN (GGG) cDNA for construct 2278.

[1211] The DNA encoding the synthetic single-chain form of INSULIN (GGG) was PCR generated using four overlapping primers. The sequence corresponding to the C-peptide in the middle region of the proinsulin cDNA was replaced by the synthetic linker "GG^{32}GPGKR" (SEQ ID NO:2148) to avoid the need for proinsulin processing and to ensure proper folding of the single-chain protein. The sequence was codon optimized for expression in yeast *S. cerevisiae*. The PCR fragment was digested with *Sal* I/*Cla* I and subcloned into *Xho* I/*Cla* I digested pScCHSA. A *Not* I fragment was then subcloned into the pSAC35 plasmid. Construct ID #2278 encodes for the chimeric leader sequence of HSA fused to the amino-terminus of the synthetic single-chain form of INSULIN (GGG) followed by the mature form of HSA.

[1212] The 5' and 3' primers of the four overlapping oligonucleotides suitable for PCR amplification of the polynucleotide encoding the synthetic single-chain form of INSULIN (GGG), INSULIN (GGG)-7 and INSULIN (GGG)-8, were synthesized:

INSULIN (GGG)-7: 5'-TCCAGGAGC*GTCGAC*AAAAGA**TTCGTTAACCAACACTTG TGTGGTTCTCACTTGGTTGAAGCTTTGTACTTGGTTTG TGGTGAA** -3'
(SEQ ID NO:903)

INSULIN (GGG)-8: 5'-AGACTTTAA*ATCGAT*GAGCAACCTCACTCTTGTGTGCATC**G TTACAGTAGTTTTCCAATTGGTACAAAGAACAAATAGAAG TACAA**–3' (SEQ ID NO:904)

[1213] INSULIN (GGG)-7 incorporates a *Sal* I cloning site (shown in italics) and the DNA encoding the first 21 amino acids (shown in bold) of the ORF of the synthetic single-chain form of INSULIN(GGG). In INSULIN (GGG)-8, the italicized sequence is a *Cla* I site; and the *Cla* I site and the DNA following it are the reverse complement of DNA encoding the first 10 amino acids of the mature HSA protein. The bolded sequence is the reverse complement of the 46 nucleotides encoding the last 15 amino acid residues Cys-44 to Asn-58 of the synthetic single-chain form of INSULIN (GGG). With these two primers, the synthetic single-chain INSULIN (GGG) protein was generated by annealing, extension of the annealed primers, digestion with *Sal* I and *Cla* I, and subcloning into *Xho* I/*Cla* I digested pScCHSA. The *Not* I fragment from this clone was then ligated into the *Not* I site of pSAC35 to generate construct ID 2278. Construct ID #2278 encodes an albumin fusion protein containing the chimeric leader sequence, the synthetic single-chain form of INSULIN (GGG), and the mature form of HSA.

[1214] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing should confirm the presence of the expected INSULIN (GGG) sequence (see below).

[1215] INSULIN albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the synthetic single-chain analog of INSULIN, i.e., Phe-1 to Asn–58; the sequence corresponding to the C-peptide in the middle region of the proinsulin cDNA was replaced by the synthetic linker "$GG^{32}GPGKR$" (SEQ ID NO:2148). In one embodiment of the invention, INSULIN albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature INSULIN albumin fusion protein is secreted directly into the culture medium. INSULIN albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, INSULIN albumin fusion proteins of the invention comprise the native INSULIN. In further preferred embodiments, the INSULIN albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2278.

Expression in yeast S. cerevisiae.

[1216] Construct 2278 can be transformed into yeast *S. cerevisiae* by methods known in the art (see Example 3). Expression levels can be examined by immunoblot detection with anti-HSA serum as the primary antibody.

Purification from yeast S. cerevisiae cell supernatant.

[1217] The cell supernatant containing the secreted INSULIN (GGG) albumin fusion protein expressed from construct ID #2278 in yeast *S. cerevisiae* can be purified as described in Example 4. N-terminal sequencing of the expressed and purified albumin fusion protein should generate FVNQH (SEQ ID NO:2149) which corresponds to the amino terminus of the synthetic single-chain long-acting insulin analog (INSULIN ($GG^{32}G$)).

In vitro [^{3}H]-2-Deoxyglucose Uptake Assay in the presence of the albumin fusion protein encoded by construct 2278.

Method

[1218] The *in vitro* assay to measure the glucose uptake in 3T3-L1 adipocytes in the presence of the INSULIN (GGG) albumin fusion protein encoded by construct 2278 can be carried out as described below in Example 41. Other assays known in the art that may be used to test INSULIN (GGG) albumin fusion proteins' include, but are not limited to, L6 Rat Myoblast Proliferation Assay via glycogen synthase kinase-3 (GSK-3) and H4IIe reporter assays (see Example 48) including the rat Malic Enzyme Promoter (rMEP)-SEAP, Sterol Regulatory Element Binding Protein (SREBP)-SEAP, Fatty Acid Synthetase (FAS)-SEAP, and PhosphoEnolPyruvate CarboxyKinase (PEPCK)-SEAP reporters.

In vitro Pancreatic Cell-lines Proliferation Assay in the presence of the albumin fusion protein encoded by construct 2278.

Method

[1219] The *in vitro* assay to measure the differentiation and proliferation of ductal epithelium pancreatic ARIP cell-line into insulin-producing beta cells and/or to measure the proliferation of the insulin-producing RIN-M beta cell-line in the presence of the INSULIN (GGG) albumin fusion protein encoded by construct 2278 can be carried out as described below under heading: "Example 42: *In vitro* Assay of [^{3}H]-Thymidine Incorporation into Pancreatic Cell-lines".

The activity of the albumin fusion protein encoded by construct 2278 can be assayed in vivo using diabetic NOD and/or NIDDM mouse models.

[1220] The activity of the INSULIN (GGG) albumin fusion protein encoded by construct 2278 can be measured using NOD and/or NIDDM mouse models described below under the headings, "Example 44: Occurrence of Diabetes in NOD Mice", "Example 45: Histological Examination of NOD Mice", and "Example 47: *In vivo* Mouse Model of NIDDM".

Example 37: Indications for Insulin albumin fusion proteins.

[1221] Results from *in vitro* assays described above indicate that insulin albumin

fusion proteins are useful for the treatment, prevention, and/or diagnosis of hyperglycemia, insulin resistance, insulin deficiency, hyperlipidemia, hyperketonemia, and diabetes mellitus, Type 1 and Type 2 diabetes.

Example 38: Preparation of HSA-hGH Fusion Proteins.

[1222] An HSA-hGH fusion protein was prepared as follows:

Cloning of hGH cDNA

[1223] The hGH cDNA was obtained from a human pituitary gland cDNA library (catalogue number HL1097v, Clontech Laboratories, Inc) by PCR amplification. Two oligonucleotides suitable for PCR amplification of the hGH cDNA, HGH1 and HGH2, were synthesized using an Applied Biosystems 380B Oligonucleotide Synthesizer.

HGH1: 5' - CCCAAGAATTCCCTTATCCAGGC - 3' (SEQ ID NO:1020)

HGH2: 5' - GGGAAGCTTAGAAGCCACAGGATCCCTCCACAG - 3' (SEQ ID NO:1021)

[1224] HGH 1 and HGH2 differed from the equivalent portion of the hGH cDNA sequence (Martial *et. al.,* 1979) by two and three nucleotides, respectively, such that after PCR amplification an *Eco*RI site would be introduced to the 5' end of the cDNA and a *Bam*H1 site would be introduced into the 3' end of the cDNA. In addition, HGH2 contained a *Hind*III site immediately downstream of the hGH sequence.

[1225] PCR amplification using a Perkin-Elmer-Cetus Thermal Cycler 9600 and a Perkin-Elmer-Cetus PCR kit, was performed using single-stranded DNA template isolated from the phage particles of the cDNA library as follows: 10 µL phage particles were lysed by the addition of 10 µL phage lysis buffer (280 µg/mL proteinase K in TE buffer) and incubation at 55°C for 15 min followed by 85°C for 15 min. After a 1 min. incubation on ice, phage debris was pelleted by centrifugation at 14,000 rpm for 3 min. The PCR mixture contained 6 µL of this DNA template, 0.1 µM of each primer and 200 µM of each deoxyribonucleotide. PCR was carried out for 30 cycles, denaturing at 94°C for 30 s, annealing at 65°C for 30 s and extending at 72°C for 30 s, increasing the extension time by 1 s per cycle.

[1226] Analysis of the reaction by gel electrophoresis showed a single product of the expected size (589 base pairs).

[1227] The PCR product was purified using Wizard PCR Preps DNA Purification

System (Promega Corp) and then digested with *Eco*Rl and *Hind*III. After further purification of the *Eco*Rl-*Hind*III fragment by gel electrophoresis, the product was cloned into pUC19 (GIBCO BRL) digested with *Eco*RI and *Hind*III, to give pHGH1. DNA sequencing of the *Eco*Rl *Hind*III region showed that the PCR product was identical in sequence to the hGH sequence (Martial *et al.*, 1979), except at the 5' and 3' ends, where the *Eco*Rl and *Bam*HI sites had been introduced, respectively.

Expression of the hGH cDNA.

[1228] The polylinker sequence of the phagemid pBluescribe (+) (Stratagene) was replaced by inserting an oligonucleotide linker, formed by annealing two 75-mer oligonucleotides, between the *Eco*RI and *Hind*III sites to form pBST(+). The new polylinker included a unique *Not*I site.

[1229] The *Not*I HSA expression cassette of pAYE309 (EP 431 880) comprising the PRBI promoter, DNA encoding the HSA/MFα-1 hybrid leader sequence, DNA encoding HSA and the ADH1 terminator, was transferred to pBST(+) to form pHSA1. The HSA coding sequence was removed from this plasmid by digestion with *Hind* III followed by religation to form pHSA2.

[1230] Cloning of the hGH cDNA provided the hGH coding region lacking the pro-hGH sequence and the first 8 base pairs (bp) of the mature hGH sequence. In order to construct an expression plasmid for secretion of hGH from yeast, a yeast promoter, signal peptide and the first 8 bp of the hGH sequence were attached to the 5' end of the cloned hGH sequence as follows: The *Hind*III-*Sfa*NI fragment from pHSA 1 was attached to the 5' end of the *Eco*RI/*Hind*III fragment from pHGHI via two synthetic oligonucleotides, HGH3 and HGH4 (which can anneal to one another in such a way as to generate a double stranded fragment of DNA with sticky ends that can anneal with *Sfa*NIand *Eco*RI sticky ends):

HGH3: 5' - GATAAAGATTCCCAAC - 3' (SEQ ID NO:1023)

HGH4: 5' - AATTGTTGGGAATCTTT- 3' (SEQ ID NO:1024)

[1231] The *Hind* III fragment so formed was cloned into *Hind*III-digested pHSA2 to make pHGH2, such that the hGH cDNA was positioned downstream of the PRBI promoter and HSA/MFα-1 fusion leader sequence (see, International Publication No. WO 90/01063). The *Not*I expression cassette contained in pHGH2, which included the *ADH1* terminator downstream of the hGH cDNA, was cloned into *Not*I-digested pSAC35 (Sleep *et al.*, BioTechnology 8:42 (1990)) to make pHGH12. This plasmid comprised the entire 2 μm plasmid to provide replication functions and the LEU2 gene for selection of transformants.

[1232] pHGH12 was introduced into *S. cerevisiae* D88 by transformation and individual transformants were grown for 3 days at 30°C in 10 mL YEPD (1% w/v yeast extract, 2 % w/v, peptone, 2 % w/v, dextrose).

[1233] After centrifugation of the cells, the supernatants were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were found to contain protein which was of the expected size and which was recognized by anti-hGH antiserum (Sigma, Poole, UK) on Western blots.

Cloning and expression of an HSA-hGH fusion protein.

[1234] In order to fuse the *HSA* cDNA to the 5' end of the hGH cDNA, the pHSA1 *Hind*III-*Bsu*361 fragment (containing most of the *HSA* cDNA) was joined to the pHGH1 *Eco*RI-*Hind*III fragment (containing most of the hGH cDNA) via two oligonucleotides, HGH7 and HGH8

HGH7: 5' - TTAGGCTTATTCCCAAC 3' (SEQ ID NO:1025)

HGH8: 5' - AATTGTTGGGAATAAGCC 3' (SEQ ID NO:1026)

[1235] The *Hind*III fragment so formed was cloned into pHSA2 digested with *Hind*III to make pHGH10, and the *Not*I expression cassette of this plasmid was cloned into *Not*I-digested pSAC35 to make pHGH16.

[1236] pHGH16 was used to transform *S. cerevisiae* D88 and supernatants of cultures were analyzed as described above. A predominant band was observed that had a molecular weight of approximately 88 kD, corresponding to the combined masses of HSA and hGH. Western blotting using anti-*HSA* and anti-hGH antisera (Sigma) confirmed the presence of the two constituent parts of the albumin fusion protein.

[1237] The albumin fusion protein was purified from culture supernatant by cation exchange chromatography, followed by anion exchange and gel permeation chromatography. Analysis of the N-terminus of the protein by amino acid sequencing confirmed the presence of the expected albumin sequence.

[1238] An *in vitro* growth hormone activity assay (Ealey *et al.*, Growth Regulation 5:36 (1995)) indicated that the albumin fusion protein possessed full hGH activity. In a hypophysectomised rat weight gain model, performed essentially as described in the European Pharmacopoeia (1987, monograph 556), the fusion molecule was more potent than hGH when the same number of units of activity (based on the above *in vitro* assay) were administered daily. Further experiments in which the albumin fusion protein was administered once every four days showed a similar overall growth response to a daily

administration of hGH. Pharmacokinetic experiments in which ^{125}I- labeled protein was administered to rats indicated an approximately ten-fold increase in circulatory half-life for the albumin fusion protein compared to hGH.

[1239] A similar plasmid was constructed in which DNA encoding the *S. cerevisiae* invertase (SUC2) leader sequence replaced the sequence for the hybrid leader, such that the encoded leader and the junction (↓) with the HSA sequence were as follows:

... MLLQAFLFLLAGFAAKISA ↓ DAHKS ..... (SEQ ID NO:1027)

Invertase leader *HSA* sequence ...

[1240] On introduction into *S. cerevisiae* DBI, this plasmid directed the expression and secretion of the albumin fusion protein at a level similar to that obtained with pHGH16. Analysis of the N-terminus of the albumin fusion protein indicated precise and efficient cleavage of the leader sequence from the mature protein.

Cloning and expression of an hGH-HSA fusion protein.

[1241] In order to fuse the hGH cDNA to the 5' end of the HSA cDNA, the HSA cDNA was first altered by site-directed mutagenesis to introduce an *Eco*NI site near the 5' end of the coding region. This was done by the method of Kunkel *et al.* (Methods in Enzymol. 154:367 (1987)) using single-stranded DNA template prepared from pHSAI and a synthetic oligonucleotide, LEU4:

LEU4: 5' - GAGATGCACACCTGAGTGAGG - 3' (SEQ ID NO:1028)

Site-directed mutagenesis using this oligonucleotide changed the coding sequence of the HSA cDNA from Lys4 to Leu4 (K4L). However, this change was repaired when the hGH cDNA was subsequently joined at the 5' end by linking the pHGH2 *Not*I-*Bam*HI fragment to the *Eco*NI-*Not*I fragment of the mutated pHSAI, via the two oligonucleotides HGH5 and HGH6:

HGH5: 5' - GATCCTGTGGCTTCGATGCACACAAGA - 3' (SEQ ID NO:1029)

HGH6: 5' - CTCTTGTGTGCATCGAAGCCACAG - 3' (SEQ ID NO:1030)

[1242] The *Not*I fragment so formed was cloned into *Not*I-digested pSAC35 to make pHGH14. pHGH14 was used to transform S. cerevisiae D88 and supernatants of culture were analyzed as above. A predominant band was observed that had a molecular weight of approximately 88 kD, corresponding to the combined masses of hGH and HSA. Western blotting using anti-HSA and anti-hGH antisera confirmed the presence of the two constituent parts of the albumin fusion protein.

[1243] The albumin fusion protein was purified from culture supernatant by cation

exchange chromatography, followed by anion exchange and gel permeation chromatography. Analysis of the N-terminus of the protein by amino acid sequencing confirmed the presence of the expected hGH sequence.

[1244] *In vitro* studies showed that the albumin fusion protein retained hGH activity, but was significantly less potent than an albumin fusion protein comprising full length HSA (1-585) as the N-terminal portion and hGH as the C-terminal portion, as described above.

Construction of plasmids for the expression of hGH fusions to domains of HSA.

[1245] Fusion polypeptides were made in which the hGH molecule was fused to the first two domains of HSA (residues 1 to 387). Fusion to the N terminus of hGH was achieved by joining the pHSA1 *Hind*III-*Sap*I fragment, which contained most of the coding sequence for domains 1 and 2 of HSA, to the pHGHI *Eco*RI-*Hind*III fragment, via the oligonucleotides HGH 11 and HGH 12:

HGH11: 5' - TGTGGAAGAGCCTCAGAATTTATTCCCAAC - 3' (SEQ ID NO:1031)

HGH12: 5' - AATTGTTGGGAATAAATTCTGAGGCTCTTCC - 3' (SEQ ID NO:1032)

[1246] The *Hind*III fragment so formed was cloned into *Hind*III-digested pHSA2 to make pHGH37 and the *Not*I expression cassette of this plasmid was cloned into *Not*I-digested pSAC35.

[1247] The resulting plasmid, pHGH38, contained an expression cassette that was found to direct secretion of the fusion polypeptide into the supernatant when transformed into S. cerevisiae DB 1. Western blotting using anti-*HSA* and anti-hGH antisera confirmed the presence of the two constituent parts of the albumin fusion protein.

[1248] The albumin fusion protein was purified from culture supernatant by cation exchange chromatography followed by gel permeation chromatography.

[1249] *In vivo* studies with purified protein indicated that the circulatory half-life was longer than that of hGH, and similar to that of an albumin fusion protein comprising full-length HSA (1-585) as the N-terminal portion and hGH as the C-terminal portion, as described above. *In vitro* studies showed that the albumin fusion protein retained hGH activity.

[1250] Using a similar strategy as detailed above, an albumin fusion protein comprising the first domain of HSA (residues 1-194) as the N-terminal portion and hGH as the C-terminal portion, was cloned and expressed in *S. cerevisiae* DBL. Western blotting of culture supernatant using anti-*HSA* and anti-hGH antisera confirmed the presence of the two constituent parts of the albumin fusion protein.

Fusion of HSA to hGH using a flexible linker sequence

[1251] Flexible linkers, comprising repeating units of $[Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser]_n$, (SEQ ID NO:2150) where n was either 2 or 3, were introduced between the *HSA* and hGH albumin fusion protein by cloning of the oligonucleotides HGH16, HGH17, HGH18 and HGH19:

HGH16:5'-TTAGGCTTAGGTGGCGGTGGATCCGGCGGTGGTGGATCTTTCCCAAC-3' (SEQ ID NO:1133)

HGH17:5'-AATTGTTGGGAAAGATCCACCACCGCCGGATCCACCGCCACCTAAGCC -3' (SEQ ID NO:1134)

HGH18:5'-TTAGGCTTAGGCGGTGGTGGATCTGGTGGCGGCGGATCTGGTGGCGGT GGATCCTTCCCAAC-3' (SEQ ID NO:1135)

HGH19:5'-AATTGTTGGGAAGGATCCACCGCCACCAGATCCGCCGCCACCAGATCC ACCACCGCCTAAGCC-3' (SEQ ID NO:1136)

[1252] Annealing of HGH16 with HGH17 resulted in n=2, while HGH18 annealed to HGH19 resulted in n=3. After annealing, the double- stranded oligonucleotides were cloned with the *Eco*RI-*Bsu*361 fragment isolated from pHGH1 into *Bsu*361-digested pHGH10 to make pHGH56 (where n=2) and pHGH57 (where n=3). The *Not*l expression cassettes from these plasmids were cloned into *Not*I-digested pSAC35 to make pHGH58 and pHGH59, respectively.

[1253] Cloning of the oligonucleotides to make pHGH56 and pHGH57 introduced a *Bam*HI site in the linker sequences. It was therefore possible to construct linker sequences in which n=1 and n = 4, by joining either the *Hind*III-*Bam*HI fragment from pHGH56 to the *Bam*HI-*Hind*III fragment from pHGH57 (making n = 1), or the *Hind*III-*Bam*HI fragment from pHGH57 to the *Bam*HI-*Hind*III fragment from pHGH56 (making n=2). Cloning of these fragments into the *Hind* III site of pHSA2, resulted in pHGH60 (n= 1) and pHGH61 (n=4). The *Not*l expression cassettes from pHGH60 and pHGH61 were cloned into *Not*l-digested pSAC35 to make pHGH62 and pHGH63, respectively.

[1254] Transformation of *S. cerevisiae* with pHGH58, pHGH59, pHGH62 and pHGH63 resulted in transformants that secreted the fusion polypeptides into the supernatant. Western blotting using anti-*HSA* and anti-hGH antisera confirmed the presence of the two constituent parts of the albumin fusion proteins.

[1255] The albumin fusion proteins were purified from culture supernatant by cation exchange chromatography, followed by anion exchange and gel permeation chromatography. Analysis of the N-termini of the proteins by amino acid sequencing confirmed the presence of

the expected albumin sequence. Analysis of the purified proteins by electrospray mass spectrometry confirmed an increase in mass of 315 D (n=l), 630 D (n=2), 945 D (n=3) and 1260 D (n=4) compared to the *HSA*-hGH fusion protein described above. The purified protein was found to be active *in vitro.*

[1256] hGH albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the mature form of hGH. In one embodiment of the invention, hGH albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature hGH albumin fusion protein is secreted directly into the culture medium. hGH albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, hGH albumin fusion proteins of the invention comprise the native hGH. In further preferred embodiments, the hGH albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Increased Shelf-Life of HSA-hGH fusion proteins: Methods

[1257] HSA-hGH and hGH were separately diluted in cell culture media containing 5% horse serum to final concentrations of 100-200 μg/ml and incubated at 4, 37 or 50°C. At time zero and at weekly intervals thereafter, aliquots of the samples were tested for their biological activity in the Nb2 cell proliferation assay, and the data normalized to the biological activity of the control (hGH solution at time zero). In other assays hGH and HSA-hGH were incubated in phosphate buffer saline in at 4, 37 and 50 degree C.

[1258] Nb2 cell proliferation assay: The growth of these cells is dependent on hGH or other lactogenic hormones. In a typical experiment 10^4 cells /well are plated in 96-well plate in the presence of different concentration of hGH or HSA-hGH in media such as DMEM containing 5-10% horse serum for 24-48 hrs in the incubator. After the incubation period, 1:10 volume of MTT (5mg/ml in H_2O) is added to each well and the plate is incubated for a further 6-16 hrs. The growing cells convert MTT to insoluble formazan. The formazan is

solublized by acidic isopropanol, and the color produced is measured at 570 nm on microtiter plate reader. The extent of formazan formation reflects the level of cellular proliferation.

Increased shelf-life of HSA-hGH fusion proteins: Results

[1259] The fusion of Therapeutic proteins to albumin confers stability in aqueous or other solution. The shelf-life of an HSA fusion protein is extended in terms of the biological activity of HSA-hGH remaining after storage in cell culture media for up to 5 weeks at 37°C. A solution of 200 μg/ml HSA-hGH was prepared in tissue culture media containing 5% horse serum, and the solution incubated at 37°C starting at time zero. At the indicated times, a sample was removed and tested for its biological activity in the Nb2 cell assay, at 2 ng/ml final concentration. The biological activity of HSA-hGH remains essentially intact (within experimental variation) after 5 weeks of incubation at 37°C. The recombinant hGH used as control for this experiment lost its biological activity in the first week of the experiment.

[1260] After storage in cell culture media for up to 3 weeks at 4, 37, or 50°C, HSA-hGH was stable. At time zero, a solution of HSA-hGH was prepared in tissue culture media containing 5% horse serum, and incubated at 4, 37, and 50°C. At the indicated periods a sample was removed and assayed for its biological activity in the Nb2 cell proliferation assay, at 60 ng/ml final concentration. HSA-hGH retains over 90% of its initial activity at all temperatures tested for at least 3 weeks after incubation while hGH loses its biological activity within the first week. This level of activity is further retained for at least 7 weeks at 37° C and 5 weeks at 50° C. These results indicate that HSA-hGH is highly stable in aqueous solution even under temperature stress.

[1261] The biological activity of HSA-hGH was stable compared to hGH in the Nb2 cell proliferation assay. Nb2 cells were grown in the presence of increasing concentrations of recombinant hGH or HSA-hGH, added at time zero. The cells were incubated for 24 or 48 hours before measuring the extent of proliferation by the MTT method. The increased stability of HSA-hGH in the assay results in essentially the same proliferative activity at 24 hours as at 48 hours while hGH shows a significant reduction in its proliferative activity after 48 hours of incubation. Compared to hGH, the HSA-hGH has lower biological potency after 1 day; the albumin fusion protein is about 5 fold less potent than hGH. However, after 2 days the HSA-hGH shows essentially the same potency as hGH due to the short life of hGH in the assay. This increase in the stability of the hGH as an albumin fusion protein has a major

unexpected impact on the biological activity of the protein.

Example 39: Indications for hGH Albumin Fusion Proteins.

[1262] Results from *in vitro* and *in vivo* assays indicate that hGH albumin fusion proteins can be used to treat, prevent, detect, diagnose; and/or ameliorate acromegaly, growth failure, growth failure and endogenous growth hormone replacement, growth hormone deficiency, growth failure or growth retardation Prader-Willi syndrome in children 2 years or older, growth deficiencies, growth failure associated with chronic renal insufficiency, postmenopausal osteoporosis, burns, cachexia, cancer cachexia, dwarfism, metabolic disorders, obesity, renal failure, Turner's Syndrome (pediatric and adult), fibromyalgia, fracture treatment, frailty, or AIDS wasting.

Example 40: Isolation of a Selected cDNA Clone From the Deposited Sample.

[1263] Many of the albumin fusion constructs of the invention have been deposited with the ATCC as shown in Table 3. The albumin fusion constructs may comprise any one of the following expression vectors: the yeast *S. cerevisiae* expression vector pSAC35, the mammalian expression vector pC4, or the mammalian expression vector pEE12.1.

[1264] pSAC35 (Sleep *et al.,* 1990, Biotechnology 8:42), pC4 (ATCC Accession No. 209646; Cullen *et al.,* Molecular and Cellular Biology, 438-447 (1985); Boshart *et al.,* Cell 41: 521-530 (1985)), and pEE12.1 (Lonza Biologics, Inc.; Stephens and Cockett, Nucl. Acids Res. 17: 7110 (1989); International Publication #WO89/01036; Murphy *et al.*, Biochem J. 227: 277-279 (1991); Bebbington *et al.,* Bio/Technology 10:169-175 (1992); US patent US 5,122,464; International Publication #WO86/05807) vectors comprise an ampicillin resistance gene for growth in bacterial cells. These vectors and/or an albumin fusion construct comprising them can be transformed into an *E. coli* strain such as Stratagene XL-1 Blue (Stratagene Cloning Systems, Inc., 11011 N. Torrey Pines Road, La Jolla, CA, 92037) using techniques described in the art such as Hanahan, spread onto Luria-Broth agar plates containing 100 μg/mL ampicillin, and grown overnight at 37 °C.

[1265] The deposited material in the sample assigned the ATCC Deposit Number cited in Table 3 for any given albumin fusion construct also may contain one or more additional albumin fusion constructs, each encoding different albumin fusion proteins. Thus,

deposits sharing the same ATCC Deposit Number contain at least an albumin fusion construct identified in the corresponding row of Table 3.

[1266] Two approaches can be used to isolate a particular albumin fusion construct from the deposited sample of plasmid DNAs cited for that albumin fusion construct in Table 3.

Method 1: Screening

[1267] First, an albumin fusion construct may be directly isolated by screening the sample of deposited plasmid DNAs using a polynucleotide probe corresponding to SEQ ID NO:X for an individual construct ID number in Table 1, using methods known in the art. For example, a specific polynucleotide with 30-40 nucleotides may be synthesized using an Applied Biosystems DNA synthesizer according to the sequence reported. The oligonucleotide can be labeled, for instance, with ^{32}P-γ-ATP using T4 polynucleotide kinase and purified according to routine methods. (E.g., Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring, NY (1982)). The albumin fusion construct from a given ATCC deposit is transformed into a suitable host, as indicated above (such as XL-1 Blue (Stratagene)) using techniques known to those of skill in the art, such as those provided by the vector supplier or in related publications or patents cited above. The transformants are plated on 1.5% agar plates (containing the appropriate selection agent, e.g., ampicillin) to a density of about 150 transformants (colonies) per plate. These plates are screened using Nylon membranes according to routine methods for bacterial colony screening (e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Edit., (1989), Cold Spring Harbor Laboratory Press, pages 1.93 to 1.104), or other techniques known to those of skill in the art.

Method 2: PCR

[1268] Alternatively, DNA encoding a given albumin fusion protein may be amplified from a sample of a deposited albumin fusion construct with SEQ ID NO:X, for example, by using two primers of 17-20 nucleotides that hybridize to the deposited albumin fusion construct 5' and 3' to the DNA encoding a given albumin fusion protein. The polymerase chain reaction is carried out under routine conditions, for instance, in 25 μl of reaction mixture with 0.5 ug of the above cDNA template. A convenient reaction mixture is 1.5-5 mM $MgCl_2$, 0.01% (w/v) gelatin, 20 μM each of dATP, dCTP, dGTP, dTTP, 25 pmol of each primer and 0.25 Unit of Taq polymerase. Thirty five cycles of PCR (denaturation at 94°C for 1 min; annealing at 55°C for 1 min; elongation at 72°C for 1 min) are performed with a

Perkin-Elmer Cetus automated thermal cycler. The amplified product is analyzed by agarose gel electrophoresis and the DNA band with expected molecular weight is excised and purified. The PCR product is verified to be the selected sequence by subcloning and sequencing the DNA product.

[1269] Several methods are available for the identification of the 5' or 3' non-coding portions of a gene which may not be present in the deposited clone. These methods include but are not limited to, filter probing, clone enrichment using specific probes, and protocols similar or identical to 5' and 3' "RACE" protocols which are known in the art. For instance, a method similar to 5' RACE is available for generating the missing 5' end of a desired full-length transcript. (Fromont-Racine et al., Nucleic Acids Res., 21(7):1683-1684 (1993)).

[1270] Briefly, a specific RNA oligonucleotide is ligated to the 5' ends of a population of RNA presumably containing full-length gene RNA transcripts. A primer set containing a primer specific to the ligated RNA oligonucleotide and a primer specific to a known sequence of the gene of interest is used to PCR amplify the 5' portion of the desired full-length gene. This amplified product may then be sequenced and used to generate the full length gene.

[1271] This above method starts with total RNA isolated from the desired source, although poly-A+ RNA can be used. The RNA preparation can then be treated with phosphatase if necessary to eliminate 5' phosphate groups on degraded or damaged RNA which may interfere with the later RNA ligase step. The phosphatase should then be inactivated and the RNA treated with tobacco acid pyrophosphatase in order to remove the cap structure present at the 5' ends of messenger RNAs. This reaction leaves a 5' phosphate group at the 5' end of the cap cleaved RNA which can then be ligated to an RNA oligonucleotide using T4 RNA ligase.

[1272] This modified RNA preparation is used as a template for first strand cDNA synthesis using a gene specific oligonucleotide. The first strand synthesis reaction is used as a template for PCR amplification of the desired 5' end using a primer specific to the ligated RNA oligonucleotide and a primer specific to the known sequence of the gene of interest. The resultant product is then sequenced and analyzed to confirm that the 5' end sequence belongs to the desired gene.

Example 41: [^{3}H]-2-Deoxyglucose Uptake Assay.

[1273] Adipose, skeletal muscle, and liver are insulin-sensitive tissues. Insulin can stimulate glucose uptake/transport into these tissues. In the case of adipose and skeletal

muscle, insulin initiates the signal transduction that eventually leads to the translocation of the glucose transporter 4 molecule, GLUT4, from a specialized intracellular compartment to the cell surface. Once on the cell surface, GLUT4 allows for glucose uptake/transport.

[^{3}H]-2-Deoxyglucose Uptake

[1274] A number of adipose and muscle related cell-lines can be used to test for glucose uptake/transport activity in the absence or presence of a combination of any one or more of the therapeutic drugs listed for the treatment of diabetes mellitus. In particular, the 3T3-L1 murine fibroblast cells and the L6 murine skeletal muscle cells can be differentiated into 3T3-L1 adipocytes and into myotubes, respectively, to serve as appropriate *in vitro* models for the [^{3}H]-2-deoxyglucose uptake assay (Urso et al., J Biol Chem, 274(43): 30864-73 (1999); Wang et al., J Mol Endocrinol, 19(3): 241-8 (1997); Haspel et al., J Membr Biol, 169 (1): 45-53 (1999); Tsakiridis et al., Endocrinology, 136(10): 4315-22 (1995)). Briefly, 2 x 10^5 cells/100 µL of adipocytes or differentiated L6 cells are transferred to 96-well Tissue-Culture, "TC", treated, i.e., coated with 50 µg/mL of poly-L-lysine, plates in post-differentiation medium and are incubated overnight at 37 °C in 5% CO_2. The cells are first washed once with serum free low glucose DMEM medium and are then starved with 100 µL/well of the same medium and with 100 µL/well of either buffer or of a combination of any one or more of the therapeutic drugs listed for the treatment of diabetes mellitus, for example, increasing concentrations of 1 nM, 10 nM, and 100 nM of the therapeutics of the subject invention (e.g., specific fusions disclosed as SEQ ID NO:Y and fragments and variants thereof) for 16 hours at 37 °C in the absence or presence of 1 nM insulin. The plates are washed three times with 100 µL/well of HEPES buffered saline. Insulin is added at 1 nM in HEPES buffered saline for 30 min at 37 °C in the presence of 10 µM labeled [^{3}H]-2-deoxyglucose (Amersham, #TRK672) and 10 µM unlabeled 2-deoxyglucose (SIGMA, D-3179). As control, the same conditions are carried out except in the absence of insulin. A final concentration of 10 µM cytochalasin B (SIGMA, C6762) is added at 100 µL/well in a separate well to measure the non-specific uptake. The cells are washed three times with HEPES buffered saline. Labeled, i.e., 10 µM of [^{3}H]-2-deoxyglucose, and unlabeled, i.e., 10 µM of 2-deoxyglucose, are added for 10 minutes at room temperature. The cells are washed three times with cold Phosphate Buffered *Sal* ine, "PBS". The cells are lysed upon the addition of 150 µL/well of 0.2 N NaOH and subsequent incubation with shaking for 20 minutes at room temperature. Samples are then transferred to a scintillation vial to which is

added 5 mL of scintillation fluid. The vials are counted in a Beta-Scintillation counter. Uptake in duplicate conditions, the difference being the absence or presence of insulin, is determined with the following equation: [(Insulin counts per minute "cpm" – Non-Specific cpm)/(No Insulin cpm – Non-Specific cpm)]. Average responses fall within the limits of about 5-fold and 3-fold that of controls for adipocytes and myotubes, respectively.

Differentiation of Cells

[1275] The cells are allowed to become fully confluent in a T-75 cm^2 flask. The medium is removed and replaced with 25 mL of pre-differentiation medium for 48 hours. The cells are incubated at 37 °C, in 5% CO_2, 85% humidity. After 48 hours, the pre-differentiation medium is removed and replaced with 25 mL differentiation medium for 48 hours. The cells are again incubated at 37 °C, in 5% CO_2, 85% humidity. After 48 hours, the medium is removed and replaced with 30 mL post-differentiation medium. Post-differentiation medium is maintained for 14-20 days or until complete differentiation is achieved. The medium is changed every 2-3 days. Human adipocytes can be purchased from Zen-Bio, INC (# SA-1096).

Example 42: *In vitro* Assay of [^{3}H]-Thymidine Incorporation into Pancreatic Cell-lines.

[1276] It has recently been shown that GLP-1 induces differentiation of the rat pancreatic ductal epithelial cell-line ARIP in a time- and dose-dependent manner which is associated with an increase in Islet Duodenal Homeobox-1 (IDX-1) and insulin mRNA levels (Hui et al., 2001, Diabetes, 50(4): 785-96). The IDX-1 in turn increases mRNA levels of the GLP-1 receptor.

Cells Types Tested

[1277] RIN-M cells: These cells are available from the American Type Tissue Culture Collection (ATCC Cell Line Number CRL-2057). The RIN-M cell line was derived from a radiation induced transplantable rat islet cell tumor. The line was established from a nude mouse xenograft of the tumor. The cells produce and secrete islet polypeptide hormones, and produce L-dopa decarboxylase (a marker for cells having amine precursor uptake and decarboxylation, or APUD, activity).

[1278] ARIP cells: These are pancreatic exocrine cells of epithelial morphology available from the American Type Tissue Culture Collection (ATCC Cell Line Number CRL-1674). See also, references: Jessop, N.W. and Hay, R.J., "Characteristics of two rat

pancreatic exocrine cell lines derived from transplantable tumors," In Vitro 16: 212, (1980); Cockell, M. et al., "Identification of a cell-specific DNA-binding activity that interacts with a transcriptional activator of genes expressed in the acinar pancreas," Mol. Cell. Biol. 9: 2464-2476, (1989); Roux, E., et al. "The cell-specific transcription factor PTF1 contains two different subunits that interact with the DNA" Genes Dev. 3: 1613-1624, (1989); and, Hui, H., et al., "Glucagon-like peptide 1 induces differentiation of islet duodenal homeobox-1-positive pancreatic ductal cells into insulin-secreting cells," Diabetes 50: 785-796 (2001).

Preparation of Cells

[1279] The RIN-M cell-line is grown in RPMI 1640 medium (Hyclone, #SH300027.01) with 10% fetal bovine serum (HyClone, #SH30088.03) and is subcultured every 6 to 8 days at a ratio of 1:3 to 1:6. The medium is changed every 3 to 4 days.

[1280] The ARIP (ATCC #CRL-1674) cell-line is grown in Ham's F12K medium (ATCC, #30-2004) with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 10% fetal bovine serum. The ARIP cell-line is subcultured at a ratio of 1:3 to 1:6 twice per week. The medium is changed every 3 to 4 days.

Assay Protocol

[1281] The cells are seeded at 4000 cells/well in 96-well plates and cultured for 48 to 72 hours to 50% confluence. The cells are switched to serum-free media at 100 μL/well. After incubation for 48-72 hours, serum and/or the therapeutics of the subject invention (e.g., albumin fusion proteins of the invention and fragments and variants thereof) are added to the well. Incubation persists for an additional 36 hours. [^{3}H]-Thymidine (5-20 Ci/mmol) (Amersham Pharmacia, #TRK120) is diluted to 1 microCuries/5 microliters. After the 36 hour incubation, 5 microliters is added per well for a further 24 hours. The reaction is terminated by washing the cells gently with cold Phosphate-Buffered *Sal* ine, "PBS", once. The cells are then fixed with 100 microliters of 10% ice cold TCA for 15 min at 4 °C. The PBS is removed and 200 microliters of 0.2 N NaOH is added. The plates are incubated for 1 hour at room temperature with shaking. The solution is transferred to a scintillation vial and 5 mL of scintillation fluid compatible with aqueous solutions is added and mixed vigorously. The vials are counted in a beta scintillation counter. As negative control, only buffer is used. As a positive control fetal calf serum is used.

Example 43: Assaying for Glycosuria.

[1282] Glycosuria (i.e., excess sugar in the urine), can be readily assayed to provide an index of the disease state of diabetes mellitus. Excess urine in a patient sample as compared with a normal patient sample is symptomatic of IDDM and NIDDM. Efficacy of treatment of such a patient having IDDM and NIDDM is indicated by a resulting decrease in the amount of excess glucose in the urine. In a preferred embodiment for IDDM and NIDDM monitoring, urine samples from patients are assayed for the presence of glucose using techniques known in the art. Glycosuria in humans is defined by a urinary glucose concentration exceeding 100 mg per 100 ml. Excess sugar levels in those patients exhibiting glycosuria can be measured even more precisely by obtaining blood samples and assaying serum glucose.

Example 44: Occurrence of Diabetes in NOD Mice.

[1283] Female NOD (non-obese diabetic) mice are characterized by displaying IDDM with a course which is similar to that found in humans, although the disease is more pronounced in female than male NOD mice. Hereinafter, unless otherwise stated, the term "NOD mouse" refers to a female NOD mouse. NOD mice have a progressive destruction of beta cells which is caused by a chronic autoimmune disease. Thus, NOD mice begin life with euglycemia, or normal blood glucose levels. By about 15 to 16 weeks of age, however, NOD mice start becoming hyperglycemic, indicating the destruction of the majority of their pancreatic beta cells and the corresponding inability of the pancreas to produce sufficient insulin. Thus, both the cause and the progression of the disease are similar to human IDDM patients.

[1284] *In vivo* assays of efficacy of the immunization regimens can be assessed in female NOD/LtJ mice (commercially available from The Jackson Laboratory, Bar Harbor, Me.). In the literature, it's reported that 80% of female mice develop diabetes by 24 weeks of age and onset of insulitis begins between 6-8 weeks age. NOD mice are inbred and highly responsive to a variety of immunoregulatory strategies. Adult NOD mice (6-8 weeks of age) have an average mass of 20-25 g.

[1285] These mice can be either untreated (control), treated with the therapeutics of the subject invention (e.g., albumin fusion proteins of the invention and fragments and variants thereof), alone or in combination with other therapeutic compounds stated above. The effect of these various treatments on the progression of diabetes can be measured as

follows:

[1286] At 14 weeks of age, the female NOD mice can be phenotyped according to glucose tolerance. Glucose tolerance can be measured with the intraperitoneal glucose tolerance test (IPGTT). Briefly, blood is drawn from the paraorbital plexus at 0 minutes and 60 minutes after the intraperitoneal injection of glucose (1 g/kg body weight). Normal tolerance is defined as plasma glucose at 0 minutes of less than 144 mg %, or at 60 minutes of less than 160 mg %. Blood glucose levels are determined with a Glucometer Elite apparatus.

[1287] Based upon this phenotypic analysis, animals can be allocated to the different experimental groups. In particular, animals with more elevated blood glucose levels can be assigned to the impaired glucose tolerance group. The mice can be fed ad libitum and can be supplied with acidified water (pH 2.3).

[1288] The glucose tolerant and intolerant mice can be further subdivided into control, albumin fusion proteins of the subject invention, and albumin fusion proteins/therapeutic compounds combination groups. Mice in the control group can receive an interperitoneal injection of vehicle daily, six times per week. Mice in the albumin fusion group can receive an interperitoneal injection of the therapeutics of the subject invention (e.g., albumin fusion proteins of the invention and fragments and variants thereof) in vehicle daily, six times per week. Mice in the albumin fusion proteins/therapeutic compounds combination group can receive both albumin fusion proteins and combinations of therapeutic compounds as described above.

[1289] The level of urine glucose in the NOD mice can be determined on a bi-weekly basis using Labstix (Bayer Diagnostics, Hampshire, England). Weight and fluid intake can also be determined on a bi-weekly basis. The onset of diabetes is defined after the appearance of glucosuria on two consecutive determinations. After 10 weeks of treatment, an additional IPGTT can be performed and animals can be sacrificed the following day.

[1290] Over the 10 week course of treatment, control animals in both the glucose tolerant and glucose intolerant groups develop diabetes at a rate of 60% and 86%, respectively (see US patent No. 5,866,546, Gross et al.). Thus, high rates of diabetes occur even in NOD mice which are initially glucose tolerant if no intervention is made.

[1291] Results can be confirmed by the measurement of blood glucose levels in NOD mice, before and after treatment. Blood glucose levels are measured as described above in both glucose tolerant and intolerant mice in all groups described.

[1292] In an alternative embodiment, the therapeutics of the subject invention (e.g., specific fusions disclosed as SEQ ID NO:Y and fragments and variants thereof) can be quantified using spectrometric analysis and appropriate protein quantities can be resuspended prior to injection in 50 .mu.l phosphate buffered saline (PBS) per dose. Two injections, one week apart, can be administered subcutaneously under the dorsal skin of each mouse. Monitoring can be performed on two separate occasions prior to immunization and can be performed weekly throughout the treatment and continued thereafter. Urine can be tested for glucose every week (Keto-Diastix.RTM.; Miles Inc., Kankakee, Ill.) and glycosuric mice can be checked for serum glucose (ExacTech.RTM., MediSense, Inc., Waltham, Mass.). Diabetes is diagnosed when fasting glycemia is greater than 2.5g/L.

Example 45: Histological Examination of NOD Mice.

[1293] Histological examination of tissue samples from NOD mice can demonstrate the ability of the compositions of the present invention, and/or a combination of the compositions of the present invention with other therapeutic agents for diabetes, to increase the relative concentration of beta cells in the pancreas. The experimental method is as follows:

[1294] The mice from Example 44 can be sacrificed at the end of the treatment period and tissue samples can be taken from the pancreas. The samples can be fixed in 10% formalin in 0.9% saline and embedded in wax. Two sets of 5 serial 5 .mu.m sections can be cut for immunolabelling at a cutting interval of 150 .mu.m. Sections can be immunolabelled for insulin (guinea pig anti-insulin antisera dilution 1:1000, ICN Thames U.K.) and glucagon (rabbit anti-pancreatic glucagon antisera dilution 1:2000) and detected with peroxidase conjugated anti-guinea pig (Dako, High Wycombe, U.K.) or peroxidase conjugated anti-rabbit antisera (dilution 1:50, Dako).

[1295] The composition of the present invention may or may not have as strong an effect on the visible mass of beta cells as it does on the clinical manifestations of diabetes in glucose tolerant and glucose intolerant animals.

Example 46: Pancreatic Beta-Cell Transplantation Combination Therapy.

[1296] Transplantation is a common form of treatment of autoimmune disease, especially when the target self tissue has been severely damaged. For example, and not by way of limitation, pancreas transplantation and islet cell transplantation are common

treatment options for IDDM (See, e.g., Stewart et al., Journal of Clinical Endocrinology & Metabolism 86 (3): 984-988 (2001); Brunicardi, Transplant. Proc. 28: 2138-40 (1996); Kendall & Robertson, Diabetes Metab. 22: 157-163 (1996); Hamano et al., Kobe J. Med. Sci. 42: 93-104 (1996); Larsen & Stratta, Diabetes Metab. 22: 139-146 (1996); and Kinkhabwala, et al., Am. J. Surg. 171: 516-520 (1996)). As with any transplantation method, transplantation therapies for autoimmune disease patients include treatments to minimize the risk of host rejection of the transplanted tissue. However, autoimmune disease involves the additional, independent risk that the pre-existing host autoimmune response which damaged the original self tissue will exert the same damaging effect on the transplanted tissue. Accordingly, the present invention encompasses methods and compositions for the treatment of autoimmune pancreatic disease using the albumin fusion proteins of the subject invetion in combination with immunomodulators/immunosuppressants in individuals undergoing transplantation therapy of the autoimmune disease.

[1297] In accordance with the invention, the albumin fusion-based compositions and formulations described above, are administered to prevent and treat damage to the transplanted organ, tissue, or cells resulting from the host individual's autoimmune response initially directed against the original self tissue. Administration may be carried out both prior and subsequent to transplantation in 2 to 4 doses each one week apart.

[1298] The following immunomodulators/immunosuppressants including, but not limited to, AI-401, CDP-571 (anti-TNF monoclonal antibody), CG-1088, Diamyd (diabetes vaccine), ICM3 (anti-ICAM-3 monoclonal antibody), linomide (Roquinimex), NBI-6024 (altered peptide ligand), TM-27, VX-740 (HMR-3480), caspase 8 protease inhibitors, thalidomide, hOKT3gamma1 (Ala-ala) (anti-CD3 monoclonal antibody), Oral Interferon-Alpha, oral lactobacillus, and LymphoStat-B™ can be used together with the albumin fusion therapeutics of the subject invention in islet cell or pancreas transplantation.

Example 47: *In vivo* Mouse Model of NIDDM.

[1299] Male C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME) can be obtained at 3 weeks of age and fed on conventional chow or diets enriched in either fat (35.5% wt/wt; Bioserv.Frenchtown, NJ) or fructose (60% wt/wt; Harlan Teklad, Madison, WI). The regular chow is composed of 4.5% wt/wt fat, 23% wt/wt protein, 31.9% wt/wt starch, 3.7% wt/wt fructose, and 5.3% wt/wt fiber. The high-fat (lard) diet is composed of 35.5% wt/wt fat, 20% wt/wt protein, 36.4% wt/wt starch, 0.0% wt/wt fructose, and 0.1%

wt/wt fiber. The high-fructose diet is composed of 5% wt/wt fat, 20% wt/wt protein, 0.0% wt/wt starch, 60% wt/wt fructose, and 9.4% wt/wt fiber. The mice may be housed no more than five per cage at 22° +/- 3°C temperature- and 50% +/- 20% humidity-controlled room with a 12-hour light (6 am to 6 pm)/dark cycle (Luo et al., 1998, Metabolism 47(6): 663-8, "Nongenetic mouse models of non-insulin-dependent diabetes mellitus"; Larsen et al., Diabetes 50(11): 2530-9 (2001), "Systemic administration of the long-acting GLP-1 derivative NN2211 induces lasting and reversible weight loss in both normal and obese rats"). After exposure to the respective diets for 3 weeks, mice can be injected intraperitoneally with either streptozotocin, "STZ" (Sigma, St. Louis, MO), at 100 mg/kg body weight or vehicle (0.05 mol/L citric acid, pH 4.5) and kept on the same diet for the next 4 weeks. Under nonfasting conditions, blood is obtained 1, 2, and 4 weeks post-STZ by nipping the distal part of the tail. Samples are used to measure nonfasting plasma glucose and insulin concentrations. Body weight and food intake are recorded weekly.

[1300] To directly determine the effect of the high-fat diet on the ability of insulin to stimulate glucose disposal, the experiments can be initiated on three groups of mice, fat-fed, chow-fed injected with vehicle, and fat-fed injected with STZ at the end of the 7-week period described above. Mice can be fasted for 4 hours before the experiments. In the first series of experiments, mice can be anesthetized with methoxyflurane (Pitman-Moor, Mundelein, IL) inhalation. Regular insulin (Sigma) can be injected intravenously ([IV] 0.1 U/kg body weight) through a tail vein, and blood can be collected 3, 6, 9, 12, and 15 minutes after the injection from a different tail vein. Plasma glucose concentrations can be determined on these samples, and the half-life ($t_{1/2}$) of glucose disappearance from plasma can be calculated using WinNonlin (Scientific Consulting, Apex, NC), a pharmacokinetics/pharmacodynamics software program.

[1301] In the second series of experiments, mice can be anesthetized with intraperitoneal sodium pentobarbital (Sigma). The abdominal cavity is opened, and the main abdominal vein is exposed and catheterized with a 24-gauge IV catheter (Johnson-Johnson Medical, Arlington, TX). The catheter is secured to muscle tissue adjacent to the abdominal vein, cut on the bottom of the syringe connection, and hooked to a prefilled PE50 plastic tube, which in turn is connected to a syringe with infusion solution. The abdominal cavity is then sutured closed. With this approach, there would be no blockage of backflow of the blood from the lower part of the body. Mice can be infused continuously with glucose (24.1 mg/kg/min) and insulin (10 mU/kg/min) at an infusion volume of 10 μL/min. Retro-orbital

blood samples (70 μL each) can be taken 90, 105, 120, and 135 minutes after the start of infusion for measurement of plasma glucose and insulin concentrations. The mean of these four samples is used to estimate steady-state plasma glucose (SSPG) and insulin (SSPI) concentrations for each animal.

[1302] Finally, experiments to evaluate the ability of the albumin fusion proteins, the therapeutic compositions of the instant application, either alone or in combination with any one or more of the therapeutic drugs listed for the treatment of diabetes mellitus, to decrease plasma glucose can be performed in the following two groups of "NIDDM" mice models that are STZ-injected: (1) fat-fed C57BL/6J, and (2) fructose-fed C57BL/6J. Plasma glucose concentrations of the mice for these studies may range from 255 to 555 mg/dL. Mice are randomly assigned to treatment with either vehicle, albumin fusion therapeutics of the present invention either alone or in combination with any one or more of the therapeutic drugs listed for the treatment of diabetes mellitus. A total of three doses can be administered. Tail vein blood samples can be taken for measurement of the plasma glucose concentration before the first dose and 3 hours after the final dose.

[1303] Plasma glucose concentrations can be determined using the Glucose Diagnostic Kit from Sigma (Sigma No. 315), an enzyme colorimetric assay. Plasma insulin levels can be determined using the Rat Insulin RIA Kit from Linco Research (#RI-13K; St. Charles, MO).

Example 48: *In vitro* H4IIe –SEAP Reporter Assays Establishing Involvement in Insulin Action.

The Various H4IIe Reporters

[1304] H4IIe/rMEP-SEAP: The malic enzyme promoter isolated from rat (rMEP) contains a PPAR-gamma element which is in the insulin pathway. This reporter construct is stably transfected into the liver H4IIe cell-line.

[1305] H4IIe/SREBP-SEAP: The sterol regulatory element binding protein (SREBP-1c) is a transcription factor which acts on the promoters of a number of insulin-responsive genes, for example, fatty acid synthetase (FAS), and which regulates expression of key genes in fatty acid metabolism in fibroblasts, adipocytes, and hepatocytes. SREBP-1c, also known as the adipocyte determination and differentiation factor 1 (ADD-1), is considered as the primary mediator of insulin effects on gene expression in adipose cells. It's activity is modulated by the levels of insulin, sterols, and glucose. This reporter construct is stably

transfected into the liver H4IIe cell-line.

[1306] H4IIe/FAS-SEAP: The fatty acid synthetase reporter constructs contain a minimal SREBP-responsive FAS promoter. This reporter construct is stably transfected into the liver H4IIe cell-line.

[1307] H4IIe/PEPCK-SEAP: The phosphoenolpyruvate carboxykinase (PEPCK) promoter is the primary site of hormonal regulation of PEPCK gene transcription modulating PEPCK activity. PEPCK catalyzes a committed and rate-limiting step in hepatic gluconeogenesis and must therefore be carefully controlled to maintain blood glucose levels within normal limits. This reporter construct is stably transfected into the liver H4IIe cell-line.

[1308] These reporter constructs can also be stably transfected into 3T3-L1 fibroblasts and L6 myoblasts. These stable cell-lines are then differentiated into 3T3-L1 adipocytes and L6 myotubes as previously described in Example 41. The differentiated cell-lines can then be used in the SEAP assay described below.

Growth and Assay Medium

[1309] The growth medium comprises 10% Fetal Bovine Serum (FBS), 10% Calf Serum, 1% NEAA, 1x penicillin/streptomycin, and 0.75 mg/mL G418 (for H4IIe/rFAS-SEAP and H4IIe/SREBP-SEAP) or 0.50 mg/mL G418 (for H4IIe/rMEP-SEAP). For H4IIe/PEPCK-SEAP, the growth medium consists of 10% FBS, 1% penicillin/streptomycin, 15 mM HEPES buffered saline, and 0.50 mg/mL G418.

[1310] The assay medium consists of low glucose DMEM medium (Life Technologies), 1% NEAA, 1x penicillin/streptomycin for the H4IIe/rFAS-SEAP, H4IIe/SREBP-SEAP, H4IIe/rMEP-SEAP reporters. The assay medium for H4IIe/PEPCK-SEAP reporter consists of 0.1% FBS, 1% penicillin/streptomycin, and 15 mM HEPES buffered saline.

Method

[1311] The 96-well plates are seeded at 75,000 cells/well in 100 μL/well of growth medium until cells in log growth phase become adherent. Cells are starved for 48 hours by replacing growth medium with assay medium, 200 μL/well. (For H4IIe/PEPCK-SEAP cells, assay medium containing 0.5 μM dexamethasone is added at 100 μL/well and incubated for approximately 20 hours). The assay medium is replaced thereafter with 100 μL/well of fresh assay medium, and a 50 μL aliquot of cell supernatant obtained from transfected cell-lines

expressing the therapeutics of the subject invention (e.g., albumin fusion proteins of the invention and fragments and variants thereof) is added to the well. Supernatants from empty vector transfected cell-lines are used as negative control. Addition of 10 nM and/or 100 nM insulin to the wells is used as positive control. After 48 hours of incubation, the conditioned media are harvested and SEAP activity measured (Phospha-Light System protocol, Tropix #BP2500). Briefly, samples are diluted 1:4 in dilution buffer and incubated at 65 °C for 30 minutes to inactivate the endogenous non-placental form of SEAP. An aliquot of 50 µL of the diluted samples is mixed with 50 µL of SEAP Assay Buffer which contains a mixture of inhibitors active against the non-placental SEAP isoenzymes and is incubated for another 5 minutes. An aliquot of 50 µL of CSPD chemiluminescent substrate which is diluted 1:20 in Emerald luminescence enhancer is added to the mixture and incubated for 15-20 minutes. Plates are read in a Dynex plate luminometer.

Example 49: Preparation of HA-cytokine or HA-growth factor fusion proteins (such as EPO, GMCSF, GCSF).

[1312] The cDNA for the cytokine or growth factor of interest, such as EPO, can be isolated by a variety of means including from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. The nucleotide sequences for all of these proteins are known and available, for instance, in U.S. Patents 4,703,008, 4,810,643 and 5,908,763. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. EPO (or other cytokine) cDNA is cloned into a vector such as pPPC0005 (Figure 2), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines, a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 1). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 50: Preparation of HA-IFN fusion proteins (such as IFNα).

[1313] The cDNA for the interferon of interest such as IFNα can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. The nucleotide sequences for interferons, such as IFNα are known and available, for instance, in U.S. Patents 5,326,859 and 4,588,585, in EP 32 134, as well as in public databases such as GenBank. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used to clone the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus of the HA sequence, with or without the use of a spacer sequence. The IFNα (or other interferon) cDNA is cloned into a vector such as pPPC0005 (Figure 2), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 1). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Maximum protein recovery from vials

[1314] The albumin fusion proteins of the invention have a high degree of stability even when they are packaged at low concentrations. In addition, in spite of the low protein concentration, good fusion-protein recovery is observed even when the aqueous solution includes no other protein added to minimize binding to the vial walls. The recovery of vial-stored HA-IFN solutions was compared with a stock solution. 6 or 30 μg/ml HA-IFN solutions were placed in vials and stored at 4°C. After 48 or 72 hrs a volume originally equivalent to 10 ng of sample was removed and measured in an IFN sandwich ELISA. The estimated values were compared to that of a high concentration stock solution. As shown, there is essentially no loss of the sample in these vials, indicating that addition of exogenous material such as albumin is not necessary to prevent sample loss to the wall of the vials

In vivo stability and bioavailability of HA-α-IFN fusions

[1315] To determine the in vivo stability and bioavailability of a HA-α-IFN fusion

molecule, the purified fusion molecule (from yeast) was administered to monkeys. Pharmaceutical compositions formulated from HA-α-IFN fusions may account for the extended serum half-life and bioavailability. Accordingly, pharmaceutical compositions may be formulated to contain lower dosages of alpha-interferon activity compared to the native alpha-interferon molecule.

[1316] Pharmaceutical compositions containing HA-α-IFN fusions may be used to treat or prevent disease in patients with any disease or disease state that can be modulated by the administration of α-IFN. Such diseases include, but are not limited to, hairy cell leukemia, Kaposi's sarcoma, genital and anal warts, chronic hepatitis B, chronic non-A, non-B hepatitis, in particular hepatitis C, hepatitis D, chronic myelogenous leukemia, renal cell carcinoma, bladder carcinoma, ovarian and cervical carcinoma, skin cancers, recurrent respirator papillomatosis, non-Hodgkin's and cutaneous T-cell lymphomas, melanoma, multiple myeloma , AIDS, multiple sclerosis, gliobastoma, etc. (see Interferon Alpha, In: AHFS Drug Information, 1997.

[1317] Accordingly, the invention includes pharmaceutical compositions containing a HA-α-IFN fusion protein, polypeptide or peptide formulated with the proper dosage for human administration. The invention also includes methods of treating patients in need of such treatment comprising at least the step of administering a pharmaceutical composition containing at least one HA-α-IFN fusion protein, polypeptide or peptide.

Bifunctional HA-α-IFN fusions

[1318] A HA-α-IFN expression vector may be modified to include an insertion for the expression of bifunctional HA-α-IFN fusion proteins. For instance, the cDNA for a second protein of interest may be inserted in frame downstream of the "rHA-IFN" sequence after the double stop codon has been removed or shifted downstream of the coding sequence.

[1319] In one version of a bifunctional HA-α-IFN fusion protein, an antibody or fragment against B-lymphocyte stimulator protein (GenBank Acc 4455139) or polypeptide may be fused to one end of the HA component of the fusion molecule. This bifunctional protein is useful for modulating any immune response generated by the α-IFN component of the fusion.

Example 51: Preparation of HA-hormone fusion protein (such as insulin, LH, FSH).

[1320] The cDNA for the hormone of interest such as insulin can be isolated by a

variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. The nucleotide sequences for all of these proteins are known and available, for instance, in public databases such as GenBank. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The hormone cDNA is cloned into a vector such as pPPC0005 (Figure 2), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 1). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 52: Preparation of HA-soluble receptor or HA-binding protein fusion protein such as HA-TNF receptor.

[1321] The cDNA for the soluble receptor or binding protein of interest such as TNF receptor can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. The nucleotide sequences for all of these proteins are known and available, for instance, in GenBank. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The receptor cDNA is cloned into a vector such as pPPC0005 (Figure 2), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 1). This expression cassette is then excised and inserted into a plasmid

suitable for the transfection of mammalian cell lines.

Example 53: Preparation of HA-growth factors such as HA-IGF-1 fusion protein.

[1322] The cDNA for the growth factor of interest such as IGF-1 can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods (see GenBank Acc. No.NP_000609). The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The growth factor cDNA is cloned into a vector such as pPPC0005 (Figure 2), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 1). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 54: Preparation of HA-single chain antibody fusion proteins.

[1323] Single chain antibodies are produced by several methods including but not limited to: selection from phage libraries, cloning of the variable region of a specific antibody by cloning the cDNA of the antibody and using the flanking constant regions as the primer to clone the variable region, or by synthesizing an oligonucleotide corresponding to the variable region of any specific antibody. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The cell cDNA is cloned into a vector such as pPPC0005 (Figure 2), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast.

[1324] In fusion molecules of the invention, the V_H and V_L can be linked by one of the following means or a combination thereof: a peptide linker between the C-terminus of the

V_H and the N-terminus of the V_L; ; a Kex2p protease cleavage site between the V_H and V_L such that the two are cleaved apart upon secretion and then self associate; and cystine residues positioned such that the V_H and V_L can form a disulphide bond between them to link them together. An alternative option would be to place the V_H at the N-terminus of HA or an HA domain fragment and the V_L at the C-terminus of the HA or HA domain fragment.

[1325] The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 1). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines. The antibody produced in this manner can be purified from media and tested for its binding to its antigen using standard immunochemical methods.

Example 55: Preparation of HA-cell adhesion molecule fusion proteins.

[1326] The cDNA for the cell adhesion molecule of interest can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. . The nucleotide sequences for the known cell adhesion molecules are known and available, for instance, in GenBank. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The cell adhesion molecule cDNA is cloned into a vector such as pPPC0005 (Figure 2), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 1). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 56: Preparation of inhibitory factors and peptides as HA fusion proteins (such as HA-antiviral, HA-antibiotic, HA-enzyme inhibit r and HA-anti-allergic proteins).

[1327] The cDNA for the peptide of interest such as an antibiotic peptide can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The peptide cDNA is cloned into a vector such as pPPC0005 (Figure 2), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 1). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 57: Preparation of targeted HA fusion proteins.

[1328] The cDNA for the protein of interest can be isolated from cDNA library or can be made synthetically using several overlapping oligonucleotides using standard molecular biology methods. The appropriate nucleotides can be engineered in the cDNA to form convenient restriction sites and also allow the attachment of the protein cDNA to albumin cDNA similar to the method described for hGH. Also a targeting protein or peptide cDNA such as single chain antibody or peptides, such as nuclear localization signals, that can direct proteins inside the cells can be fused to the other end of albumin. The protein of interest and the targeting peptide is cloned into a vector such as pPPC0005 (Figure 2), pScCHSA, pScNHSA , or pC4:HSA which allows the fusion with albumin cDNA. In this manner both N- and C-terminal end of albumin are fused to other proteins. The fused cDNA is then excised from pPPC0005 and is inserted into a plasmid such as pSAC35 to allow the expression of the albumin fusion protein in yeast. All the above procedures can be performed using standard methods in molecular biology. The albumin fusion protein secreted from yeast can be collected and purified from the media and tested for its biological activity and its targeting activity using appropriate biochemical and biological tests.

Example 58: Preparation of HA-enzymes fusions.

[1329] The cDNA for the enzyme of interest can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The enzyme cDNA is cloned into a vector such as pPPC0005 (Figure 2), pScCHSA, pScNHSA, or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 1). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 59: Bacterial Expression of an Albumin Fusion Protein.

[1330] A polynucleotide encoding an albumin fusion protein of the present invention comprising a bacterial signal sequence is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' ends of the DNA sequence, to synthesize insertion fragments. The primers used to amplify the polynucleotide encoding insert should preferably contain restriction sites, such as BamHI and XbaI, at the 5' end of the primers in order to clone the amplified product into the expression vector. For example, BamHI and XbaI correspond to the restriction enzyme sites on the bacterial expression vector pQE-9. (Qiagen, Inc., Chatsworth, CA). This plasmid vector encodes antibiotic resistance (Amp^r), a bacterial origin of replication (ori), an IPTG-regulatable promoter/operator (P/O), a ribosome binding site (RBS), a 6-histidine tag (6-His), and restriction enzyme cloning sites.

[1331] The pQE-9 vector is digested with BamHI and XbaI and the amplified fragment is ligated into the pQE-9 vector maintaining the reading frame initiated at the bacterial RBS. The ligation mixture is then used to transform the E. coli strain M15/rep4 (Qiagen, Inc.) which contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan^r). Transformants are identified by their

ability to grow on LB plates and ampicillin/kanamycin resistant colonies are selected. Plasmid DNA is isolated and confirmed by restriction analysis.

[1332] Clones containing the desired constructs are grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells are grown to an optical density 600 ($O.D._{600}$) of between 0.4 and 0.6. IPTG (Isopropyl-B-D-thiogalacto pyranoside) is then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression.

[1333] Cells are grown for an extra 3 to 4 hours. Cells are then harvested by centrifugation (20 mins at 6000Xg). The cell pellet is solubilized in the chaotropic agent 6 Molar Guanidine HCl or preferably in 8 M urea and concentrations greater than 0.14 M 2-mercaptoethanol by stirring for 3-4 hours at 4°C (see, e.g., Burton et al., Eur. J. Biochem. *179*:379-387 (1989)). The cell debris is removed by centrifugation, and the supernatant containing the polypeptide is loaded onto a nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin column (available from QIAGEN, Inc., *supra*). Proteins with a 6 x His tag bind to the Ni-NTA resin with high affinity and can be purified in a simple one-step procedure (for details see: The QIAexpressionist (1995) QIAGEN, Inc., *supra*).

[1334] Briefly, the supernatant is loaded onto the column in 6 M guanidine-HCl, pH 8. The column is first washed with 10 volumes of 6 M guanidine-HCl, pH 8, then washed with 10 volumes of 6 M guanidine-HCl pH 6, and finally the polypeptide is eluted with 6 M guanidine-HCl, pH 5.

[1335] The purified protein is then renatured by dialyzing it against phosphate-buffered saline (PBS) or 50 mM Na-acetate, pH 6 buffer plus 200 mM NaCl. Alternatively, the protein can be successfully refolded while immobilized on the Ni-NTA column. Exemplary conditions are as follows: renature using a linear 6M-1M urea gradient in 500 mM NaCl, 20% glycerol, 20 mM Tris/HCl pH 7.4, containing protease inhibitors. The renaturation should be performed over a period of 1.5 hours or more. After renaturation the proteins are eluted by the addition of 250 mM immidazole. Immidazole is removed by a final dialyzing step against PBS or 50 mM sodium acetate pH 6 buffer plus 200 mM NaCl. The purified protein is stored at 4° C or frozen at -80° C.

[1336] In addition to the above expression vector, the present invention further includes an expression vector, called pHE4a (ATCC Accession Number 209645, deposited on February 25, 1998) which contains phage operator and promoter elements operatively

linked to a polynucleotide encoding an albumin fusion protein of the present invention, called pHE4a. (ATCC Accession Number 209645, deposited on February 25, 1998.) This vector contains: 1) a neomycinphosphotransferase gene as a selection marker, 2) an E. coli origin of replication, 3) a T5 phage promoter sequence, 4) two lac operator sequences, 5) a Shine-Delgarno sequence, and 6) the lactose operon repressor gene (lacIq). The origin of replication (oriC) is derived from pUC19 (LTI, Gaithersburg, MD). The promoter and operator sequences are made synthetically.

[1337] DNA can be inserted into the pHE4a by restricting the vector with NdeI and XbaI, BamHI, XhoI, or Asp718, running the restricted product on a gel, and isolating the larger fragment (the stuffer fragment should be about 310 base pairs). The DNA insert is generated according to PCR protocols described herein or otherwise known in the art, using PCR primers having restriction sites for NdeI (5' primer) and XbaI, BamHI, XhoI, or Asp718 (3' primer). The PCR insert is gel purified and restricted with compatible enzymes. The insert and vector are ligated according to standard protocols.

[1338] The engineered vector may be substituted in the above protocol to express protein in a bacterial system.

Example 60: Expression of an Albumin Fusion Protein in Mammalian Cells.

[1339] The albumin fusion proteins of the present invention can be expressed in a mammalian cell. A typical mammalian expression vector contains a promoter element, which mediates the initiation of transcription of mRNA, a protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription is achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g., RSV, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter).

[1340] Suitable expression vectors for use in practicing the present invention include, for example, vectors such as, pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146), pBC12MI (ATCC 67109), pCMVSport 2.0, and pCMVSport 3.0. Mammalian host cells that could be used include, but are not limited to, human Hela, 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

[1341] Alternatively, the albumin fusion protein can be expressed in stable cell lines containing the polynucleotide encoding the albumin fusion protein integrated into a chromosome. The co-transfection with a selectable marker such as DHFR, gpt, neomycin, or hygromycin allows the identification and isolation of the transfected cells.

[1342] The transfected polynucleotide encoding the fusion protein can also be amplified to express large amounts of the encoded fusion protein. The DHFR (dihydrofolate reductase) marker is useful in developing cell lines that carry several hundred or even several thousand copies of the gene of interest. (See, e.g., Alt et al., J. Biol. Chem. 253:1357-1370 (1978); Hamlin et al., Biochem. et Biophys. Acta, 1097:107-143 (1990); Page et al., Biotechnology 9:64-68 (1991)). Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy et al., Biochem J. 227:277-279 (1991); Bebbington et al., Bio/Technology 10:169-175 (1992). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of proteins.

[1343] Derivatives of the plasmid pSV2-dhfr (ATCC Accession No. 37146), the expression vectors pC4 (ATCC Accession No. 209646) and pC6 (ATCC Accession No.209647) contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen et al., Molecular and Cellular Biology, 438-447 (March, 1985)) plus a fragment of the CMV-enhancer (Boshart et al., Cell 41:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors also contain the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene, and the mouse DHFR gene under control of the SV40 early promoter.

[1344] Specifically, the plasmid pC6, for example, is digested with appropriate restriction enzymes and then dephosphorylated using calf intestinal phosphates by procedures known in the art. The vector is then isolated from a 1% agarose gel.

[1345] A polynucleotide encoding an albumin fusion protein of the present invention is generated using techniques known in the art and this polynucleotide is amplified using PCR technology known in the art. If a naturally occurring signal sequence is used to produce the fusion protein of the present invention, the vector does not need a second signal peptide. Alternatively, if a naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (See, e.g., International Publication No. WO

96/34891.)

[1346] The amplified fragment encoding the fusion protein of the invention is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with appropriate restriction enzymes and again purified on a 1% agarose gel.

[1347] The amplified fragment encoding the albumin fusion protein of the invention is then digested with the same restriction enzyme and purified on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC6 using, for instance, restriction enzyme analysis.

[1348] Chinese hamster ovary cells lacking an active DHFR gene is used for transfection. Five μg of the expression plasmid pC6 or pC4 is cotransfected with 0.5 μg of the plasmid pSVneo using lipofectin (Felgner et al., *supra*). The plasmid pSV2-neo contains a dominant selectable marker, the *neo* gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of methotrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 μM, 2 μM, 5 μM, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 200 μM. Expression of the desired fusion protein is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

Example 61: Multifusion Fusions.

[1349] The albumin fusion proteins (e.g,. containing a Therapeutic protein (or fragment or variant thereof) fused to albumin (or a fragment or variant thereof)) may additionally be fused to other proteins to generate "multifusion proteins". These multifusion proteins can be used for a variety of applications. For example, fusion of the albumin fusion proteins of the invention to His-tag, HA-tag, protein A, IgG domains, and maltose binding protein facilitates purification. (See e.g,. EP A 394,827; Traunecker et al., Nature 331:84-86

(1988)). Nuclear localization signals fused to the polypeptides of the present invention can target the protein to a specific subcellular localization, while covalent heterodimer or homodimers can increase or decrease the activity of an albumin fusion protein. Furthermore, the fusion of additional protein sequences to the albumin fusion proteins of the invention may further increase the solubility and/or stability of the fusion protein. The fusion proteins described above can be made using or routinely modifting techniques known in the art and/or by modifying the following protocol, which outlines the fusion of a polypeptide to an IgG molecule.

[1350] Briefly, the human Fc portion of the IgG molecule can be PCR amplified, using primers that span the 5' and 3' ends of the sequence described below. These primers also should have convenient restriction enzyme sites that will facilitate cloning into an expression vector, preferably a mammalian or yeast expression vector.

[1351] For example, if pC4 (ATCC Accession No. 209646) is used, the human Fc portion can be ligated into the BamHI cloning site. Note that the 3' BamHI site should be destroyed. Next, the vector containing the human Fc portion is re-restricted with BamHI, linearizing the vector, and a polynucleotide encoding an albumin fusion protein of the present invention (generateed and isolated using techniques known in the art), is ligated into this BamHI site. Note that the polynucleotide encoding the fusion protein of the invention is cloned without a stop codon, otherwise a Fc containing fusion protein will not be produced.

[1352] If the naturally occurring signal sequence is used to produce the albumin fusion protein of the present invention, pC4 does not need a second signal peptide. Alternatively, if the naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (See, e.g., International Publication No. WO 96/34891.)

Human IgG Fc region:

GGGATCCGGAGCCCAAATCTTCTGACAAAACTCACACATGCCCACCGTGCCCAG
CACCTGAATTCGAGGGTGCACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGA
CACCCTCATGATCTCCCGGACTCCTGAGGTCACATGCGTGGTGGTGGACGTAAGC
CACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCAT
AATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTC
AGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGC
AAGGTCTCCAACAAAGCCCTCCCAACCCCCATCGAGAAAACCATCTCCAAAGCC

AAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAG
CTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCAAGC
GACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGAC
CACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACC
GTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCAT
GAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAT
GAGTGCGACGGCCGCGACTCTAGAGGAT (SEQ ID NO: 1112)

Example 62: Production of an Antibody from an Albumin Fusion Protein.

Hybridoma Technology

[1353] Antibodies that bind the albumin fusion proteins of the present invention and portions of the albumin fusion proteins of the present invention (e.g., the Therapeutic protein portion or albumin portion of the fusion protein) can be prepared by a variety of methods. (See, Current Protocols, Chapter 2.) As one example of such methods, a preparation of an albumin fusion protein of the invention or a portion of an albumin fusion protein of the invention is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

[1354] Monoclonal antibodies specific for an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention, are prepared using hybridoma technology (Kohler et al., Nature 256:495 (1975); Kohler et al., Eur. J. Immunol. 6:511 (1976); Kohler et al., Eur. J. Immunol. 6:292 (1976); Hammerling et al., in: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981)). In general, an animal (preferably a mouse) is immunized with an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention. The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP2O), available from the ATCC. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands et al. (Gastroenterology 80:225-232 (1981)). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention.

[1355] Alternatively, additional antibodies capable of binding to an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention can be produced in a two-step procedure using anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the an albumin fusion protein of the invention (or portion of an albumin fusion protein of the invention) -specific antibody can be blocked by the fusion protein of the invention, or a portion of an albumin fusion protein of the invention. Such antibodies comprise anti-idiotypic antibodies to the fusion protein of the invention (or portion of an albumin fusion protein of the invention) -specific antibody and are used to immunize an animal to induce formation of further fusion protein of the invention (or portion of an albumin fusion protein of the invention) -specific antibodies.

[1356] For *in vivo* use of antibodies in humans, an antibody is "humanized". Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies described above. Methods for producing chimeric and humanized antibodies are known in the art and are discussed herein. (See, for review, Morrison, Science 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986); Cabilly et al., U.S. Patent No. 4,816,567; Taniguchi et al., EP 171496; Morrison et al., EP 173494; Neuberger et al., WO 8601533; Robinson et al., International Publication No. WO 8702671; Boulianne et al., Nature 312:643 (1984); Neuberger et al., Nature 314:268 (1985)).

[1357] *Isolation Of Antibody Fragments Directed Against an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention From A Library Of scFvs.* Naturally occurring V-genes isolated from human PBLs are constructed into a library of antibody fragments which contain reactivities against an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention, to which the donor may or may not have been exposed (see e.g., U.S. Patent 5,885,793 incorporated herein by reference in its entirety).

[1358] *Rescue of the Library.* A library of scFvs is constructed from the RNA of human PBLs as described in International Publication No. WO 92/01047. To rescue phage displaying antibody fragments, approximately 10^9 *E. coli* harboring the phagemid are used to

inoculate 50 ml of 2xTY containing 1% glucose and 100 μg/ml of ampicillin (2xTY-AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture is used to inoculate 50 ml of 2xTY-AMP-GLU, 2 x 108 TU of delta gene 3 helper (M13 delta gene III, see International Publication No. WO 92/01047) are added and the culture incubated at 37°C for 45 minutes without shaking and then at 37°C for 45 minutes with shaking. The culture is centrifuged at 4000 r.p.m. for 10 min. and the pellet resuspended in 2 liters of 2xTY containing 100 μg/ml ampicillin and 50 ug/ml kanamycin and grown overnight. Phage are prepared as described in International Publication No. WO 92/01047.

[1359] M13 delta gene III is prepared as follows: M13 delta gene III helper phage does not encode gene III protein, hence the phage(mid) displaying antibody fragments have a greater avidity of binding to antigen. Infectious M13 delta gene III particles are made by growing the helper phage in cells harboring a pUC19 derivative supplying the wild type gene III protein during phage morphogenesis. The culture is incubated for 1 hour at 37° C without shaking and then for a further hour at 37°C with shaking. Cells are spun down (IEC-Centra 8,400 r.p.m. for 10 min), resuspended in 300 ml 2xTY broth containing 100 μg ampicillin/ml and 25 μg kanamycin/ml (2xTY-AMP-KAN) and grown overnight, shaking at 37°C. Phage particles are purified and concentrated from the culture medium by two PEG-precipitations (Sambrook et al., 1990), resuspended in 2 ml PBS and passed through a 0.45 μm filter (Minisart NML; Sartorius) to give a final concentration of approximately 10^{13} transducing units/ml (ampicillin-resistant clones).

[1360] *Panning of the Library.* Immunotubes (Nunc) are coated overnight in PBS with 4 ml of either 100 μg/ml or 10 μg/ml of an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention. Tubes are blocked with 2% Marvel-PBS for 2 hours at 37°C and then washed 3 times in PBS. Approximately 10^{13} TU of phage is applied to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes are washed 10 times with PBS 0.1% Tween-20 and 10 times with PBS. Phage are eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and over turntable after which the solution is immediately neutralized with 0.5 ml of 1.0M Tris-HCl, pH 7.4. Phage are then used to infect 10 ml of mid-log E. coli TG1 by incubating eluted phage with bacteria for 30 minutes at 37°C. The E. coli are then plated on TYE plates containing 1% glucose and 100 μg/ml ampicillin. The resulting bacterial library is then rescued with delta gene 3 helper phage as described above to prepare phage for a subsequent round of selection. This process

is then repeated for a total of 4 rounds of affinity purification with tube-washing increased to 20 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

[1361] *Characterization of Binders.* Eluted phage from the 3rd and 4th rounds of selection are used to infect E. coli HB 2151 and soluble scFv is produced (Marks, et al., 1991) from single colonies for assay. ELISAs are performed with microtitre plates coated with either 10 pg/ml of an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention, in 50 mM bicarbonate pH 9.6. Clones positive in ELISA are further characterized by PCR fingerprinting (see, e.g., International Publication No. WO 92/01047) and then by sequencing. These ELISA positive clones may also be further characterized by techniques known in the art, such as, for example, epitope mapping, binding affinity, receptor signal transduction, ability to block or competitively inhibit antibody/antigen binding, and competitive agonistic or antagonistic activity.

Example 63: Method of Treatment Using Gene Therapy-Ex Vivo.

[1362] One method of gene therapy transplants fibroblasts, which are capable of expressing an albumin fusion protein of the present invention, onto a patient. Generally, fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin) is added. The flasks are then incubated at 37 degree C for approximately one week.

[1363] At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

[1364] pMV-7 (Kirschmeier, P.T. et al., DNA, 7:219-25 (1988)), flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

[1365] Polynucleotides encoding an albumin fusion protein of the invention can be generated using techniques known in the art amplified using PCR primers which correspond

to the 5' and 3' end sequences and optionally having appropriate restriction sites and initiation/stop codons, if necessary. Preferably, the 5' primer contains an EcoRI site and the 3' primer includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is then used to transform bacteria HB101, which are then plated onto agar containing kanamycin for the purpose of confirming that the vector has the gene of interest properly inserted.

[1366] The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

[1367] Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his. Once the fibroblasts have been efficiently infected, the fibroblasts are analyzed to determine whether the albumin fusion protein is produced.

[1368] The engineered fibroblasts are then transplanted onto the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads.

Example 64: Method of Treatment Using Gene Therapy - *In Vivo*.

[1369] Another aspect of the present invention is using *in vivo* gene therapy methods to treat disorders, diseases and conditions. The gene therapy method relates to the introduction of naked nucleic acid (DNA, RNA, and antisense DNA or RNA) sequences encoding an albumin fusion protein of the invention into an animal. Polynucleotides encoding albumin fusion proteins of the present invention may be operatively linked to (i.e.,

associated with) a promoter or any other genetic elements necessary for the expression of the polypeptide by the target tissue. Such gene therapy and delivery techniques and methods are known in the art, see, for example, WO90/11092, WO98/11779; U.S. Patent NO. 5693622, 5705151, 5580859; Tabata et al., Cardiovasc. Res. 35(3):470-479 (1997); Chao et al., Pharmacol. Res. 35(6):517-522 (1997); Wolff, Neuromuscul. Disord. 7(5):314-318 (1997); Schwartz et al., Gene Ther. 3(5):405-411 (1996); Tsurumi et al., Circulation 94(12):3281-3290 (1996) (incorporated herein by reference).

[1370] The polynucleotide constructs may be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, intestine and the like). The polynucleotide constructs can be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

[1371] The term "naked" polynucleotide, DNA or RNA, refers to sequences that are free from any delivery vehicle that acts to assist, promote, or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, polynucleotides encoding albumin fusion proteins of the present invention may also be delivered in liposome formulations (such as those taught in Felgner P.L. et al. (1995) Ann. NY Acad. Sci. 772:126-139 and Abdallah B. et al. (1995) Biol. Cell 85(1):1-7) which can be prepared by methods well known to those skilled in the art.

[1372] The polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Any strong promoter known to those skilled in the art can be used for driving the expression of DNA. Unlike other gene therapy techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

[1373] The polynucleotide construct can be delivered to the interstitial space of tissues within an animal, including muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers,

collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. *In vivo* muscle cells are particularly competent in their ability to take up and express polynucleotides.

[1374] For the naked polynucleotide injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 g/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration. The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked polynucleotide constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

[1375] The dose response effects of injected polynucleotide in muscle *in vivo* is determined as follows. Suitable template DNA for production of mRNA coding for polypeptide of the present invention is prepared in accordance with a standard recombinant DNA methodology. The template DNA, which may be either circular or linear, is either used as naked DNA or complexed with liposomes. The quadriceps muscles of mice are then injected with various amounts of the template DNA.

[1376] Five to six week old female and male Balb/C mice are anesthetized by intraperitoneal injection with 0.3 ml of 2.5% Avertin. A 1.5 cm incision is made on the anterior thigh, and the quadriceps muscle is directly visualized. The template DNA is injected in 0.1 ml of carrier in a 1 cc syringe through a 27 gauge needle over one minute, approximately 0.5 cm from the distal insertion site of the muscle into the knee and about 0.2 cm deep. A suture is placed over the injection site for future localization, and the skin is

closed with stainless steel clips.

[1377] After an appropriate incubation time (e.g., 7 days) muscle extracts are prepared by excising the entire quadriceps. Every fifth 15 um cross-section of the individual quadriceps muscles is histochemically stained for protein expression. A time course for fusion protein expression may be done in a similar fashion except that quadriceps from different mice are harvested at different times. Persistence of DNA in muscle following injection may be determined by Southern blot analysis after preparing total cellular DNA and HIRT supernatants from injected and control mice. The results of the above experimentation in mice can be used to extrapolate proper dosages and other treatment parameters in humans and other animals using naked DNA.

Example 65: Transgenic Animals.

[1378] The albumin fusion proteins of the invention can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, hamsters, guinea pigs, pigs, micro-pigs, goats, sheep, cows and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate transgenic animals. In a specific embodiment, techniques described herein or otherwise known in the art, are used to express fusion proteins of the invention in humans, as part of a gene therapy protocol.

[1379] Any technique known in the art may be used to introduce the polynucleotides encoding the albumin fusion proteins of the invention into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Paterson et al., Appl. Microbiol. Biotechnol. 40:691-698 (1994); Carver et al., Biotechnology (NY) 11:1263-1270 (1993); Wright et al., Biotechnology (NY) 9:830-834 (1991); and Hoppe et al., U.S. Pat. No. 4,873,191 (1989)); retrovirus mediated gene transfer into germ lines (Van der Putten et al., Proc. Natl. Acad. Sci., USA 82:6148-6152 (1985)), blastocysts or embryos; gene targeting in embryonic stem cells (Thompson et al., Cell 56:313-321 (1989)); electroporation of cells or embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814 (1983)); introduction of the polynucleotides of the invention using a gene gun (see, e.g., Ulmer et al., Science 259:1745 (1993); introducing nucleic acid constructs into embryonic pleuripotent stem cells and transferring the stem cells back into the blastocyst; and sperm-mediated gene transfer (Lavitrano et al., Cell 57:717-723 (1989); etc. For a review of such techniques, see Gordon, "Transgenic Animals," Intl. Rev. Cytol. 115:171-229 (1989), which is incorporated by reference herein in its entirety.

[1380] Any technique known in the art may be used to produce transgenic clones containing polynucleotides encoding albumin fusion proteins of the invention, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal, or adult cells induced to quiescence (Campell et al., Nature 380:64-66 (1996); Wilmut et al., Nature 385:810-813 (1997)).

[1381] The present invention provides for transgenic animals that carry the polynucleotides encoding the albumin fusion proteins of the invention in all their cells, as well as animals which carry these polynucleotides in some, but not all their cells, *i.e.,* mosaic animals or chimeric. The transgene may be integrated as a single transgene or as multiple copies such as in concatamers, *e.g.,* head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko et al., Proc. Natl. Acad. Sci. USA 89:6232-6236 (1992)). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the polynucleotide encoding the fusion protein of the invention be integrated into the chromosomal site of the endogenous gene corresponding to the Therapeutic protein portion or ablumin portion of the fusion protein of the invention, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene in only that cell type, by following, for example, the teaching of Gu et al. (Gu et al., Science 265:103-106 (1994)). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

[1382] Once transgenic animals have been generated, the expression of the recombinant gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to verify that integration of the polynucleotide encoding the fsuion protien of the invention has taken place. The level of mRNA expression of the polynucleotide encoding the fusion protein of the invention in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from

the animal, *in situ* hybridization analysis, and reverse transcriptase-PCR (rt-PCR). Samples of fusion protein-expressing tissue may also be evaluated immunocytochemically or immunohistochemically using antibodies specific for the fusion protein.

[1383] Once the founder animals are produced, they may be bred, inbred, outbred, or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include, but are not limited to: outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound transgenics that express the transgene at higher levels because of the effects of additive expression of each transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; and breeding to place the transgene (i.e., polynucleotide encoding an albumin fusion protein of the invention) on a distinct background that is appropriate for an experimental model of interest.

[1384] Transgenic animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of fusion proteins of the invention and the Therapeutic protein and/or albumin component of the fusion protein of the invention, studying conditions and/or disorders associated with aberrant expression, and in screening for compounds effective in ameliorating such conditions and/or disorders.

Example 66: Assays Detecting Stimulation or Inhibition of B cell Proliferation and Differentiation.

[1385] Generation of functional humoral immune responses requires both soluble and cognate signaling between B-lineage cells and their microenvironment. Signals may impart a positive stimulus that allows a B-lineage cell to continue its programmed development, or a negative stimulus that instructs the cell to arrest its current developmental pathway. To date, numerous stimulatory and inhibitory signals have been found to influence B cell responsiveness including IL-2, IL-4, IL-5, IL-6, IL-7, IL10, IL-13, IL-14 and IL-15. Interestingly, these signals are by themselves weak effectors but can, in combination with various co-stimulatory proteins, induce activation, proliferation, differentiation, homing, tolerance and death among B cell populations.

[1386] One of the best studied classes of B-cell co-stimulatory proteins is the TNF-superfamily. Within this family CD40, CD27, and CD30 along with their respective ligands CD154, CD70, and CD153 have been found to regulate a variety of immune responses. Assays which allow for the detection and/or observation of the proliferation and differentiation of these B-cell populations and their precursors are valuable tools in determining the effects various proteins may have on these B-cell populations in terms of proliferation and differentiation. Listed below are two assays designed to allow for the detection of the differentiation, proliferation, or inhibition of B-cell populations and their precursors.

[1387] In Vitro Assay- Albumin fusion proteins of the invention (including fusion proteins containing fragments or variants of Therapeutic proteins and/or albumin or fragments or variants of albumin) can be assessed for its ability to induce activation, proliferation, differentiation or inhibition and/or death in B-cell populations and their precursors. The activity of an albumin fusion protein of the invention on purified human tonsillar B cells, measured qualitatively over the dose range from 0.1 to 10,000 ng/mL, is assessed in a standard B-lymphocyte co-stimulation assay in which purified tonsillar B cells are cultured in the presence of either formalin-fixed *Staphylococcus aureus* Cowan I (SAC) or immobilized anti-human IgM antibody as the priming agent. Second signals such as IL-2 and IL-15 synergize with SAC and IgM crosslinking to elicit B cell proliferation as measured by tritiated-thymidine incorporation. Novel synergizing agents can be readily identified using this assay. The assay involves isolating human tonsillar B cells by magnetic bead (MACS) depletion of CD3-positive cells. The resulting cell population is greater than 95% B cells as assessed by expression of CD45R(B220) .

[1388] Various dilutions of each sample are placed into individual wells of a 96-well plate to which are added 10^5 B-cells suspended in culture medium (RPMI 1640 containing 10% FBS, 5 X 10^{-5}M 2ME, 100U/ml penicillin, 10ug/ml streptomycin, and 10^{-5} dilution of SAC) in a total volume of 150ul. Proliferation or inhibition is quantitated by a 20h pulse (1uCi/well) with 3H-thymidine (6.7 Ci/mM) beginning 72h post factor addition. The positive and negative controls are IL2 and medium respectively.

[1389] *In vivo* Assay- BALB/c mice are injected (i.p.) twice per day with buffer only, or 2 mg/Kg of an albumin fusion protein of the invention (including fusion proteins containing fragments or variants of Therapeutic proteins and/or albumin or fragments or variants of albumin). Mice receive this treatment for 4 consecutive days, at which time they

are sacrificed and various tissues and serum collected for analyses. Comparison of H&E sections from normal spleens and spleens treated with the albumin fusion protein of the invention identify the results of the activity of the fusion protein on spleen cells, such as the diffusion of peri-arterial lymphatic sheaths, and/or significant increases in the nucleated cellularity of the red pulp regions, which may indicate the activation of the differentiation and proliferation of B-cell populations. Immunohistochemical studies using a B cell marker, anti-CD45R(B220), are used to determine whether any physiological changes to splenic cells, such as splenic disorganization, are due to increased B-cell representation within loosely defined B-cell zones that infiltrate established T-cell regions.

[1390] Flow cytometric analyses of the spleens from mice treated with the albumin fusion protein is used to indicate whether the albumin fusion protein specifically increases the proportion of ThB+, CD45R(B220)dull B cells over that which is observed in control mice.

[1391] Likewise, a predicted consequence of increased mature B-cell representation *in vivo* is a relative increase in serum Ig titers. Accordingly, serum IgM and IgA levels are compared between buffer and fusion protein treated mice.

Example 67: T Cell Proliferation Assay.

[1392] A CD3-induced proliferation assay is performed on PBMCs and is measured by the uptake of ^{3}H-thymidine. The assay is performed as follows. Ninety-six well plates are coated with 100 µl/well of mAb to CD3 (HIT3a, Pharmingen) or isotype-matched control mAb (B33.1) overnight at 4 degrees C (1 µg/ml in .05M bicarbonate buffer, pH 9.5), then washed three times with PBS. PBMC are isolated by F/H gradient centrifugation from human peripheral blood and added to quadruplicate wells (5 x 10^{4}/well) of mAb coated plates in RPMI containing 10% FCS and P/S in the presence of varying concentrations of an albumin fusion protein of the invention (including fusion proteins containing fragments or variants of Therapeutic proteins and/or albumin or fragments or variants of albumin) (total volume 200 ul). Relevant protein buffer and medium alone are controls. After 48 hr. culture at 37 degrees C, plates are spun for 2 min. at 1000 rpm and 100 µl of supernatant is removed and stored –20 degrees C for measurement of IL-2 (or other cytokines) if effect on proliferation is observed. Wells are supplemented with 100 ul of medium containing 0.5 uCi of ^{3}H-thymidine and cultured at 37 degrees C for 18-24 hr. Wells are harvested and incorporation of ^{3}H-thymidine used as a measure of proliferation. Anti-CD3 alone is the positive control for proliferation. IL-2 (100 U/ml) is also used as a control which enhances

proliferation. Control antibody which does not induce proliferation of T cells is used as the negative control for the effects of fusion proteins of the invention.

Example 68: Effect of Fusion Proteins of the Invention on the Expression of MHC Class II, Costimulatory and Adhesion Molecules and Cell Differentiation of Monocytes and Monocyte-Derived Human Dendritic Cells.

[1393] Dendritic cells are generated by the expansion of proliferating precursors found in the peripheral blood: adherent PBMC or elutriated monocytic fractions are cultured for 7-10 days with GM-CSF (50 ng/ml) and IL-4 (20 ng/ml). These dendritic cells have the characteristic phenotype of immature cells (expression of CD1, CD80, CD86, CD40 and MHC class II antigens). Treatment with activating factors, such as TNF-α, causes a rapid change in surface phenotype (increased expression of MHC class I and II, costimulatory and adhesion molecules, downregulation of FCγRII, upregulation of CD83). These changes correlate with increased antigen-presenting capacity and with functional maturation of the dendritic cells.

[1394] FACS analysis of surface antigens is performed as follows. Cells are treated 1-3 days with increasing concentrations of an albumin fusion protein of the invention or LPS (positive control), washed with PBS containing 1% BSA and 0.02 mM sodium azide, and then incubated with 1:20 dilution of appropriate FITC- or PE-labeled monoclonal antibodies for 30 minutes at 4 degrees C. After an additional wash, the labeled cells are analyzed by flow cytometry on a FACScan (Becton Dickinson).

[1395] Effect on the production of cytokines. Cytokines generated by dendritic cells, in particular IL-12, are important in the initiation of T-cell dependent immune responses. IL-12 strongly influences the development of Thl helper T-cell immune response, and induces cytotoxic T and NK cell function. An ELISA is used to measure the IL-12 release as follows. Dendritic cells (10^6/ml) are treated with increasing concentrations of an albumin fusion protein of the invention for 24 hours. LPS (100 ng/ml) is added to the cell culture as positive control. Supernatants from the cell cultures are then collected and analyzed for IL-12 content using commercial ELISA kit (e.g., R & D Systems (Minneapolis, MN)). The standard protocols provided with the kits are used.

[1396] Effect on the expression of MHC Class II, costimulatory and adhesion

molecules. Three major families of cell surface antigens can be identified on monocytes: adhesion molecules, molecules involved in antigen presentation, and Fc receptor. Modulation of the expression of MHC class II antigens and other costimulatory molecules, such as B7 and ICAM-1, may result in changes in the antigen presenting capacity of monocytes and ability to induce T cell activation. Increased expression of Fc receptors may correlate with improved monocyte cytotoxic activity, cytokine release and phagocytosis.

[1397] FACS analysis is used to examine the surface antigens as follows. Monocytes are treated 1-5 days with increasing concentrations of an albumin fusion protein of the invention or LPS (positive control), washed with PBS containing 1% BSA and 0.02 mM sodium azide, and then incubated with 1:20 dilution of appropriate FITC- or PE-labeled monoclonal antibodies for 30 minutes at 4 degrees C. After an additional wash, the labeled cells are analyzed by flow cytometry on a FACScan (Becton Dickinson).

[1398] Monocyte activation and/or increased survival. Assays for molecules that activate (or alternatively, inactivate) monocytes and/or increase monocyte survival (or alternatively, decrease monocyte survival) are known in the art and may routinely be applied to determine whether a molecule of the invention functions as an inhibitor or activator of monocytes. Albumin fusion proteins of the invention can be screened using the three assays described below. For each of these assays, Peripheral blood mononuclear cells (PBMC) are purified from single donor leukopacks (American Red Cross, Baltimore, MD) by centrifugation through a Histopaque gradient (Sigma). Monocytes are isolated from PBMC by counterflow centrifugal elutriation.

[1399] Monocyte Survival Assay. Human peripheral blood monocytes progressively lose viability when cultured in absence of serum or other stimuli. Their death results from internally regulated processes (apoptosis). Addition to the culture of activating factors, such as TNF-alpha dramatically improves cell survival and prevents DNA fragmentation. Propidium iodide (PI) staining is used to measure apoptosis as follows. Monocytes are cultured for 48 hours in polypropylene tubes in serum-free medium (positive control), in the presence of 100 ng/ml TNF-alpha (negative control), and in the presence of varying concentrations of the fusion protein to be tested. Cells are suspended at a concentration of 2 x 10^6/ml in PBS containing PI at a final concentration of 5 µg/ml, and then incubated at room temperature for 5 minutes before FACScan analysis. PI uptake has been demonstrated to

correlate with DNA fragmentation in this experimental paradigm.

[1400] Effect on cytokine release. An important function of monocytes/macrophages is their regulatory activity on other cellular populations of the immune system through the release of cytokines after stimulation. An ELISA to measure cytokine release is performed as follows. Human monocytes are incubated at a density of $5x10^5$ cells/ml with increasing concentrations of an albumin fusion protein of the invention and under the same conditions, but in the absence of the fusion protein. For IL-12 production, the cells are primed overnight with IFN (100 U/ml) in the presence of the fusion protein. LPS (10 ng/ml) is then added. Conditioned media are collected after 24h and kept frozen until use. Measurement of TNF-alpha, IL-10, MCP-1 and IL-8 is then performed using a commercially available ELISA kit (e.g., R & D Systems (Minneapolis, MN)) and applying the standard protocols provided with the kit.

[1401] Oxidative burst. Purified monocytes are plated in 96-w plate at $2\text{-}1x10^5$ cell/well. Increasing concentrations of an albumin fusion protein of the invention are added to the wells in a total volume of 0.2 ml culture medium (RPMI 1640 + 10% FCS, glutamine and antibiotics). After 3 days incubation, the plates are centrifuged and the medium is removed from the wells. To the macrophage monolayers, 0.2 ml per well of phenol red solution (140 mM NaCl, 10 mM potassium phosphate buffer pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red and 19 U/ml of HRPO) is added, together with the stimulant (200 nM PMA). The plates are incubated at 37°C for 2 hours and the reaction is stopped by adding 20 μl 1N NaOH per well. The absorbance is read at 610 nm. To calculate the amount of H_2O_2 produced by the macrophages, a standard curve of a H_2O_2 solution of known molarity is performed for each experiment.

Example 69: Biological Effects of Fusion Proteins of the Invention.

Astrocyte and Neuronal Assays.

[1402] Albumin fusion proteins of the invention can be tested for activity in promoting the survival, neurite outgrowth, or phenotypic differentiation of cortical neuronal cells and for inducing the proliferation of glial fibrillary acidic protein immunopositive cells, astrocytes. The selection of cortical cells for the bioassay is based on the prevalent

expression of FGF-1 and FGF-2 in cortical structures and on the previously reported enhancement of cortical neuronal survival resulting from FGF-2 treatment. A thymidine incorporation assay, for example, can be used to elucidate an albumin fusion protein of the invention's activity on these cells.

[1403] Moreover, previous reports describing the biological effects of FGF-2 (basic FGF) on cortical or hippocampal neurons *in vitro* have demonstrated increases in both neuron survival and neurite outgrowth (Walicke et al., "Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension." *Proc. Natl. Acad. Sci. USA 83*:3012-3016. (1986), assay herein incorporated by reference in its entirety). However, reports from experiments done on PC-12 cells suggest that these two responses are not necessarily synonymous and may depend on not only which FGF is being tested but also on which receptor(s) are expressed on the target cells. Using the primary cortical neuronal culture paradigm, the ability of an albumin fusion protein of the invention to induce neurite outgrowth can be compared to the response achieved with FGF-2 using, for example, a thymidine incorporation assay.

Fibroblast and endothelial cell assays.

[1404] Human lung fibroblasts are obtained from Clonetics (San Diego, CA) and maintained in growth media from Clonetics. Dermal microvascular endothelial cells are obtained from Cell Applications (San Diego, CA). For proliferation assays, the human lung fibroblasts and dermal microvascular endothelial cells can be cultured at 5,000 cells/well in a 96-well plate for one day in growth medium. The cells are then incubated for one day in 0.1% BSA basal medium. After replacing the medium with fresh 0.1% BSA medium, the cells are incubated with the test fusion protein of the invention proteins for 3 days. Alamar Blue (Alamar Biosciences, Sacramento, CA) is added to each well to a final concentration of 10%. The cells are incubated for 4 hr. Cell viability is measured by reading in a CytoFluor fluorescence reader. For the PGE_2 assays, the human lung fibroblasts are cultured at 5,000 cells/well in a 96-well plate for one day. After a medium change to 0.1% BSA basal medium, the cells are incubated with FGF-2 or fusion protein of the invention with or without IL-1α for 24 hours. The supernatants are collected and assayed for PGE_2 by EIA kit (Cayman, Ann Arbor, MI). For the IL-6 assays, the human lung fibroblasts are cultured at 5,000 cells/well in

a 96-well plate for one day. After a medium change to 0.1% BSA basal medium, the cells are incubated with FGF-2 or with or without an albumin fusion protein of the invention and/or IL-1α for 24 hours. The supernatants are collected and assayed for IL-6 by ELISA kit (Endogen, Cambridge, MA).

[1405] Human lung fibroblasts are cultured with FGF-2 or an albumin fusion protein of the invention for 3 days in basal medium before the addition of Alamar Blue to assess effects on growth of the fibroblasts. FGF-2 should show a stimulation at 10 - 2500 ng/ml which can be used to compare stimulation with the fusion protein of the invention.

Cell proliferation based on [3H]thymidine incorporation

[1406] The following [3H]Thymidine incorporation assay can be used to measure the effect of a Therapeutic proteins, e.g., growth factor proteins, on the proliferation of cells such as fibroblast cells, epithelial cells or immature muscle cells.

[1407] Sub-confluent cultures are arrested in G1 phase by an 18 h incubation in serum-free medium. Therapeutic proteins are then added for 24 h and during the last 4 h, the cultures are labeled with [3H]thymidine, at a final concentration of 0.33 μM (25 Ci/mmol, Amersham, Arlington Heights, IL). The incorporated [3H]thymidine is precipitated with ice-cold 10% trichloroacetic acid for 24 h. Subsequently, the cells are rinsed sequentially with ice-cold 10% trichloroacetic acid and then with ice-cold water. Following lysis in 0.5 M NaOH, the lysates and PBS rinses (500 ml) are pooled, and the amount of radioactivity is measured.

Parkinson Models.

[1408] The loss of motor function in Parkinson's disease is attributed to a deficiency of striatal dopamine resulting from the degeneration of the nigrostriatal dopaminergic projection neurons. An animal model for Parkinson's that has been extensively characterized involves the systemic administration of 1-methyl-4 phenyl 1,2,3,6-tetrahydropyridine (MPTP). In the CNS, MPTP is taken-up by astrocytes and catabolized by monoamine oxidase B to 1-methyl-4-phenyl pyridine (MPP^+) and released. Subsequently, MPP^+ is actively accumulated in dopaminergic neurons by the high-affinity reuptake transporter for dopamine. MPP^+ is then concentrated in mitochondria by the electrochemical gradient and selectively inhibits nicotidamide adenine disphosphate: ubiquinone oxidoreductionase

(complex I), thereby interfering with electron transport and eventually generating oxygen radicals.

[1409] It has been demonstrated in tissue culture paradigms that FGF-2 (basic FGF) has trophic activity towards nigral dopaminergic neurons (Ferrari et al., Dev. Biol. 1989). Recently, Dr. Unsicker's group has demonstrated that administering FGF-2 in gel foam implants in the striatum results in the near complete protection of nigral dopaminergic neurons from the toxicity associated with MPTP exposure (Otto and Unsicker, J. Neuroscience, 1990).

[1410] Based on the data with FGF-2, an albumin fusion protein of the invention can be evaluated to determine whether it has an action similar to that of FGF-2 in enhancing dopaminergic neuronal survival *in vitro* and it can also be tested *in vivo* for protection of dopaminergic neurons in the striatum from the damage associated with MPTP treatment. The potential effect of an albumin fusion protein of the invention is first examined in vitro in a dopaminergic neuronal cell culture paradigm. The cultures are prepared by dissecting the midbrain floor plate from gestation day 14 Wistar rat embryos. The tissue is dissociated with trypsin and seeded at a density of 200,000 cells/cm^2 on polyorthinine-laminin coated glass coverslips. The cells are maintained in Dulbecco's Modified Eagle's medium and F12 medium containing hormonal supplements (N1). The cultures are fixed with paraformaldehyde after 8 days in vitro and are processed for tyrosine hydroxylase, a specific marker for dopaminergic neurons, immunohistochemical staining. Dissociated cell cultures are prepared from embryonic rats. The culture medium is changed every third day and the factors are also added at that time.

[1411] Since the dopaminergic neurons are isolated from animals at gestation day 14, a developmental time which is past the stage when the dopaminergic precursor cells are proliferating, an increase in the number of tyrosine hydroxylase immunopositive neurons would represent an increase in the number of dopaminergic neurons surviving *in vitro*. Therefore, if a therapeutic protein of the invention acts to prolong the survival of dopaminergic neurons, it would suggest that the fusion protein may be involved in Parkinson's Disease.

Example 70: The Effect of Albumin Fusion Proteins of the Invention on the Growth of Vascular Endothelial Cells.

[1412] On day 1, human umbilical vein endothelial cells (HUVEC) are seeded at 2-

$5x10^4$ cells/35 mm dish density in M199 medium containing 4% fetal bovine serum (FBS), 16 units/ml heparin, and 50 units/ml endothelial cell growth supplements (ECGS, Biotechnique, Inc.). On day 2, the medium is replaced with M199 containing 10% FBS, 8 units/ml heparin. An albumin fusion protein of the invention, and positive controls, such as VEGF and basic FGF (bFGF) are added, at varying concentrations. On days 4 and 6, the medium is replaced. On day 8, cell number is determined with a Coulter Counter.

[1413] An increase in the number of HUVEC cells indicates that the fusion protein may proliferate vascular endothelial cells, while a decrease in the number of HUVEC cells indicates that the fusion protein inhibits vascular endothelial cells.

Example 71: Rat Corneal Wound Healing Model.

[1414] This animal model shows the effect of an albumin fusion protein of the invention on neovascularization. The experimental protocol includes:

Making a 1-1.5 mm long incision from the center of cornea into the stromal layer.

Inserting a spatula below the lip of the incision facing the outer corner of the eye.

Making a pocket (its base is 1-1.5 mm form the edge of the eye).

Positioning a pellet, containing 50ng- 5ug of an albumin fusion protein of the invention, within the pocket.

[1415] Treatment with an an albumin fusion protein of the invention can also be applied topically to the corneal wounds in a dosage range of 20mg - 500mg (daily treatment for five days).

Example 72: Diabetic Mouse and Glucocorticoid-Impaired Wound Healing Models.

Diabetic db+/db+ Mouse Model.

[1416] To demonstrate that an albumin fusion protein of the invention accelerates the healing process, the genetically diabetic mouse model of wound healing is used. The full thickness wound healing model in the db+/db+ mouse is a well characterized, clinically relevant and reproducible model of impaired wound healing. Healing of the diabetic wound is dependent on formation of granulation tissue and re-epithelialization rather than contraction (Gartner, M.H. *et al., J. Surg. Res. 52*:389 (1992); Greenhalgh, D.G. *et al., Am. J. Pathol. 136*:1235 (1990)).

[1417] The diabetic animals have many of the characteristic features observed in Type II diabetes mellitus. Homozygous (db+/db+) mice are obese in comparison to their normal

heterozygous (db+/+m) littermates. Mutant diabetic (db+/db+) mice have a single autosomal recessive mutation on chromosome 4 (db+) (Coleman *et al. Proc. Natl. Acad. Sci. USA 77*:283-293 (1982)). Animals show polyphagia, polydipsia and polyuria. Mutant diabetic mice (db+/db+) have elevated blood glucose, increased or normal insulin levels, and suppressed cell-mediated immunity (Mandel *et al., J. Immunol. 120*:1375 (1978); Debray-Sachs, M. *et al., Clin. Exp. Immunol. 51(1)*:1-7 (1983); Leiter *et al., Am. J. of Pathol. 114*:46-55 (1985)). Peripheral neuropathy, myocardial complications, and microvascular lesions, basement membrane thickening and glomerular filtration abnormalities have been described in these animals (Norido, F. *et al., Exp. Neurol. 83(2)*:221-232 (1984); Robertson *et al., Diabetes 29(1)*:60-67 (1980); Giacomelli *et al., Lab Invest. 40(4)*:460-473 (1979); Coleman, D.L., *Diabetes 31 (Suppl)*:1-6 (1982)). These homozygous diabetic mice develop hyperglycemia that is resistant to insulin analogous to human type II diabetes (Mandel *et al., J. Immunol. 120*:1375-1377 (1978)).

[1418] The characteristics observed in these animals suggests that healing in this model may be similar to the healing observed in human diabetes (Greenhalgh, *et al., Am. J. of Pathol. 136*:1235-1246 (1990)).

[1419] Genetically diabetic female C57BL/KsJ (db+/db+) mice and their non-diabetic (db+/+m) heterozygous littermates are used in this study (Jackson Laboratories). The animals are purchased at 6 weeks of age and are 8 weeks old at the beginning of the study. Animals are individually housed and received food and water ad libitum. All manipulations are performed using aseptic techniques. The experiments are conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

[1420] Wounding protocol is performed according to previously reported methods (Tsuboi, R. and Rifkin, D.B., *J. Exp. Med. 172*:245-251 (1990)). Briefly, on the day of wounding, animals are anesthetized with an intraperitoneal injection of Avertin (0.01 mg/mL), 2,2,2-tribromoethanol and 2-methyl-2-butanol dissolved in deionized water. The dorsal region of the animal is shaved and the skin washed with 70% ethanol solution and iodine. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is then created using a Keyes tissue punch. Immediately following wounding, the surrounding skin is gently stretched to eliminate wound expansion. The wounds are left open for the duration of the experiment. Application of the treatment is given topically for 5 consecutive days commencing on the day of wounding. Prior to treatment,

wounds are gently cleansed with sterile saline and gauze sponges.

[1421] Wounds are visually examined and photographed at a fixed distance at the day of surgery and at two day intervals thereafter. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

[1422] An albumin fusion protein of the invention is administered using at a range different doses, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

[1423] Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology and immunohistochemistry. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

[1424] Three groups of 10 animals each (5 diabetic and 5 non-diabetic controls) are evaluated: 1) Vehicle placebo control, 2) untreated group, and 3) treated group.

[1425] Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total square area of the wound. Contraction is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 is 64mm^2, the corresponding size of the dermal punch. Calculations are made using the following formula:

a. [Open area on day 8] - [Open area on day 1] / [Open area on day 1]

[1426] Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using a Reichert-Jung microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds are used to assess whether the healing process and the morphologic appearance of the repaired skin is altered by treatment with an albumin fusion protein of the invention. This assessment included verification of the presence of cell accumulation, inflammatory cells, capillaries, fibroblasts, re-epithelialization and epidermal maturity (Greenhalgh, D.G. *et al., Am. J. Pathol. 136:*1235 (1990)). A calibrated lens micrometer is used by a blinded observer.

[1427] Tissue sections are also stained immunohistochemically with a polyclonal

rabbit anti-human keratin antibody using ABC Elite detection system. Human skin is used as a positive tissue control while non-immune IgG is used as a negative control. Keratinocyte growth is determined by evaluating the extent of reepithelialization of the wound using a calibrated lens micrometer.

[1428] Proliferating cell nuclear antigen/cyclin (PCNA) in skin specimens is demonstrated by using anti-PCNA antibody (1:50) with an ABC Elite detection system. Human colon cancer served as a positive tissue control and human brain tissue is used as a negative tissue control. Each specimen included a section with omission of the primary antibody and substitution with non-immune mouse IgG. Ranking of these sections is based on the extent of proliferation on a scale of 0-8, the lower side of the scale reflecting slight proliferation to the higher side reflecting intense proliferation.

[1429] Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

Steroid Impaired Rat Model

[1430] The inhibition of wound healing by steroids has been well documented in various *in vitro* and *in vivo* systems (Wahl, Glucocorticoids and Wound healing. In: Anti-Inflammatory Steroid Action: Basic and Clinical Aspects. 280-302 (1989); Wahl*et al., J. Immunol. 115*: 476-481 (1975); Werb *et al., J. Exp. Med. 147*:1684-1694 (1978)). Glucocorticoids retard wound healing by inhibiting angiogenesis, decreasing vascular permeability (Ebert *et al., An. Intern. Med. 37*:701-705 (1952)), fibroblast proliferation, and collagen synthesis (Beck *et al., Growth Factors. 5:* 295-304 (1991); Haynes *et al., J. Clin. Invest. 61:* 703-797 (1978)) and producing a transient reduction of circulating monocytes (Haynes et al., *J. Clin. Invest. 61:* 703-797 (1978); Wahl, "Glucocorticoids and wound healing", *In:* Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989)). The systemic administration of steroids to impaired wound healing is a well establish phenomenon in rats (Beck *et al., Growth Factors. 5:* 295-304 (1991); Haynes *et al., J. Clin. Invest. 61:* 703-797 (1978); Wahl, "Glucocorticoids and wound healing", *In*: Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989); Pierce *et al., Proc. Natl. Acad. Sci. USA 86*: 2229-2233 (1989)).

[1431] To demonstrate that an albumin fusion protein of the invention can accelerate the healing process, the effects of multiple topical applications of the fusion protein on full

thickness excisional skin wounds in rats in which healing has been impaired by the systemic administration of methylprednisolone is assessed.

[1432] Young adult male Sprague Dawley rats weighing 250-300 g (Charles River Laboratories) are used in this example. The animals are purchased at 8 weeks of age and are 9 weeks old at the beginning of the study. The healing response of rats is impaired by the systemic administration of methylprednisolone (17mg/kg/rat intramuscularly) at the time of wounding. Animals are individually housed and received food and water *ad libitum*. All manipulations are performed using aseptic techniques. This study is conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

[1433] The wounding protocol is followed according to that described above. On the day of wounding, animals are anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The dorsal region of the animal is shaved and the skin washed with 70% ethanol and iodine solutions. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is created using a Keyes tissue punch. The wounds are left open for the duration of the experiment. Applications of the testing materials are given topically once a day for 7 consecutive days commencing on the day of wounding and subsequent to methylprednisolone administration. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

[1434] Wounds are visually examined and photographed at a fixed distance at the day of wounding and at the end of treatment. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

[1435] The fusion protein of the invention is administered using at a range different doses, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

[1436] Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

[1437] Three groups of 10 animals each (5 with methylprednisolone and 5 without glucocorticoid) are evaluated: 1) Untreated group 2) Vehicle placebo control 3) treated

groups.

[1438] Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total area of the wound. Closure is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 is $64mm^2$, the corresponding size of the dermal punch. Calculations are made using the following formula:

a. [Open area on day 8] - [Open area on day 1] / [Open area on day 1]

[1439] Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using an Olympus microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds allows assessment of whether the healing process and the morphologic appearance of the repaired skin is improved by treatment with an albumin fusion protein of the invention. A calibrated lens micrometer is used by a blinded observer to determine the distance of the wound gap.

[1440] Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

Example 73: Lymphedema Animal Model.

[1441] The purpose of this experimental approach is to create an appropriate and consistent lymphedema model for testing the therapeutic effects of an albumin fusion protein of the invention in lymphangiogenesis and re-establishment of the lymphatic circulatory system in the rat hind limb. Effectiveness is measured by swelling volume of the affected limb, quantification of the amount of lymphatic vasculature, total blood plasma protein, and histopathology. Acute lymphedema is observed for 7-10 days. Perhaps more importantly, the chronic progress of the edema is followed for up to 3-4 weeks.

[1442] Prior to beginning surgery, blood sample is drawn for protein concentration analysis. Male rats weighing approximately ~350g are dosed with Pentobarbital. Subsequently, the right legs are shaved from knee to hip. The shaved area is swabbed with gauze soaked in 70% EtOH. Blood is drawn for serum total protein testing. Circumference and volumetric measurements are made prior to injecting dye into paws after marking 2 measurement levels (0.5 cm above heel, at mid-pt of dorsal paw). The intradermal dorsum of

both right and left paws are injected with 0.05 ml of 1% Evan's Blue. Circumference and volumetric measurements are then made following injection of dye into paws.

[1443] Using the knee joint as a landmark, a mid-leg inguinal incision is made circumferentially allowing the femoral vessels to be located. Forceps and hemostats are used to dissect and separate the skin flaps. After locating the femoral vessels, the lymphatic vessel that runs along side and underneath the vessel(s) is located. The main lymphatic vessels in this area are then electrically coagulated or suture ligated.

[1444] Using a microscope, muscles in back of the leg (near the semitendinosis and adductors) are bluntly dissected. The popliteal lymph node is then located. The 2 proximal and 2 distal lymphatic vessels and distal blood supply of the popliteal node are then ligated by suturing. The popliteal lymph node, and any accompanying adipose tissue, is then removed by cutting connective tissues.

[1445] Care is taken to control any mild bleeding resulting from this procedure. After lymphatics are occluded, the skin flaps are sealed by using liquid skin (Vetbond) (AJ Buck). The separated skin edges are sealed to the underlying muscle tissue while leaving a gap of ~0.5 cm around the leg. Skin also may be anchored by suturing to underlying muscle when necessary.

[1446] To avoid infection, animals are housed individually with mesh (no bedding). Recovering animals are checked daily through the optimal edematous peak, which typically occurred by day 5-7. The plateau edematous peak are then observed. To evaluate the intensity of the lymphedema, the circumference and volumes of 2 designated places on each paw before operation and daily for 7 days are measured. The effect of plasma proteins on lymphedema is determined and whether protein analysis is a useful testing perimeter is also investigated. The weights of both control and edematous limbs are evaluated at 2 places. Analysis is performed in a blind manner.

[1447] Circumference Measurements: Under brief gas anesthetic to prevent limb movement, a cloth tape is used to measure limb circumference. Measurements are done at the ankle bone and dorsal paw by 2 different people and those 2 readings are averaged. Readings are taken from both control and edematous limbs.

[1448] Volumetric Measurements: On the day of surgery, animals are anesthetized with Pentobarbital and are tested prior to surgery. For daily volumetrics animals are under brief halothane anesthetic (rapid immobilization and quick recovery), and both legs are shaved and equally marked using waterproof marker on legs. Legs are first dipped in water,

then dipped into instrument to each marked level then measured by Buxco edema software(Chen/Victor). Data is recorded by one person, while the other is dipping the limb to marked area.

[1449] Blood-plasma protein measurements: Blood is drawn, spun, and serum separated prior to surgery and then at conclusion for total protein and $Ca2^{+}$ comparison.

[1450] Limb Weight Comparison: After drawing blood, the animal is prepared for tissue collection. The limbs are amputated using a quillitine, then both experimental and control legs are cut at the ligature and weighed. A second weighing is done as the tibio-cacaneal joint is disarticulated and the foot is weighed.

[1451] Histological Preparations: The transverse muscle located behind the knee (popliteal) area is dissected and arranged in a metal mold, filled with freezeGel, dipped into cold methylbutane, placed into labeled sample bags at - 80EC until sectioning. Upon sectioning, the muscle is observed under fluorescent microscopy for lymphatics..

Example 74: Suppression of TNF alpha-Induced Adhesion Molecule Expression by an Albumin Fusion Protein of the Invention.

[1452] The recruitment of lymphocytes to areas of inflammation and angiogenesis involves specific receptor-ligand interactions between cell surface adhesion molecules (CAMs) on lymphocytes and the vascular endothelium. The adhesion process, in both normal and pathological settings, follows a multi-step cascade that involves intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) expression on endothelial cells (EC). The expression of these molecules and others on the vascular endothelium determines the efficiency with which leukocytes may adhere to the local vasculature and extravasate into the local tissue during the development of an inflammatory response. The local concentration of cytokines and growth factor participate in the modulation of the expression of these CAMs.

[1453] Tumor necrosis factor alpha (TNF-a), a potent proinflammatory cytokine, is a stimulator of all three CAMs on endothelial cells and may be involved in a wide variety of inflammatory responses, often resulting in a pathological outcome.

[1454] The potential of an albumin fusion protein of the invention to mediate a suppression of TNF-a induced CAM expression can be examined. A modified ELISA assay which uses ECs as a solid phase absorbent is employed to measure the amount of CAM expression on TNF-a treated ECs when co-stimulated with a member of the FGF family of

proteins.

[1455] To perform the experiment, human umbilical vein endothelial cell (HUVEC) cultures are obtained from pooled cord harvests and maintained in growth medium (EGM-2; Clonetics, San Diego, CA) supplemented with 10% FCS and 1% penicillin/streptomycin in a 37 degree C humidified incubator containing 5% CO_2. HUVECs are seeded in 96-well plates at concentrations of 1 x 10^4 cells/well in EGM medium at 37 degree C for 18-24 hrs or until confluent. The monolayers are subsequently washed 3 times with a serum-free solution of RPMI-1640 supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, and treated with a given cytokine and/or growth factor(s) for 24 h at 37 degree C. Following incubation, the cells are then evaluated for CAM expression.

[1456] Human Umbilical Vein Endothelial cells (HUVECs) are grown in a standard 96 well plate to confluence. Growth medium is removed from the cells and replaced with 90 ul of 199 Medium (10% FBS). Samples for testing and positive or negative controls are added to the plate in triplicate (in 10 ul volumes). Plates are incubated at 37 degree C for either 5 h (selectin and integrin expression) or 24 h (integrin expression only). Plates are aspirated to remove medium and 100 μl of 0.1% paraformaldehyde-PBS(with Ca^{++} and Mg^{++}) is added to each well. Plates are held at 4°C for 30 min.

[1457] Fixative is then removed from the wells and wells are washed 1X with PBS(+Ca,Mg)+0.5% BSA and drained. Do not allow the wells to dry. Add 10 μl of diluted primary antibody to the test and control wells. Anti-ICAM-1-Biotin, Anti-VCAM-1-Biotin and Anti-E-selectin-Biotin are used at a concentration of 10 μg/ml (1:10 dilution of 0.1 mg/ml stock antibody). Cells are incubated at 37°C for 30 min. in a humidified environment. Wells are washed X3 with PBS(+Ca,Mg)+0.5% BSA.

[1458] Then add 20 μl of diluted ExtrAvidin-Alkaline Phosphotase (1:5,000 dilution) to each well and incubated at 37°C for 30 min. Wells are washed X3 with PBS(+Ca,Mg)+0.5% BSA. 1 tablet of p-Nitrophenol Phosphate pNPP is dissolved in 5 ml of glycine buffer (pH 10.4). 100 μl of pNPP substrate in glycine buffer is added to each test well. Standard wells in triplicate are prepared from the working dilution of the ExtrAvidin-Alkaline Phosphotase in glycine buffer: 1:5,000 (10^0) > $10^{-0.5}$ > 10^{-1} > $10^{-1.5}$. 5 μl of each dilution is added to triplicate wells and the resulting AP content in each well is 5.50 ng, 1.74 ng, 0.55 ng, 0.18 ng. 100 μl of pNNP reagent must then be added to each of the standard wells. The plate must be incubated at 37°C for 4h. A volume of 50 μl of 3M NaOH is added

to all wells. The results are quantified on a plate reader at 405 nm. The background subtraction option is used on blank wells filled with glycine buffer only. The template is set up to indicate the concentration of AP-conjugate in each standard well [5.50 ng; 1.74 ng; 0.55 ng; 0.18 ng]. Results are indicated as amount of bound AP-conjugate in each sample.

Example 75: Construction of GAS Reporter Construct.

[1459] One signal transduction pathway involved in the differentiation and proliferation of cells is called the Jaks-STATs pathway. Activated proteins in the Jaks-STATs pathway bind to gamma activation site "GAS" elements or interferon-sensitive responsive element ("ISRE"), located in the promoter of many genes. The binding of a protein to these elements alter the expression of the associated gene.

[1460] GAS and ISRE elements are recognized by a class of transcription factors called Signal Transducers and Activators of Transcription, or "STATs." There are six members of the STATs family. Stat1 and Stat3 are present in many cell types, as is Stat2 (as response to IFN-alpha is widespread). Stat4 is more restricted and is not in many cell types though it has been found in T helper class I, cells after treatment with IL-12. Stat5 was originally called mammary growth factor, but has been found at higher concentrations in other cells including myeloid cells. It can be activated in tissue culture cells by many cytokines.

[1461] The STATs are activated to translocate from the cytoplasm to the nucleus upon tyrosine phosphorylation by a set of kinases known as the Janus Kinase ("Jaks") family. Jaks represent a distinct family of soluble tyrosine kinases and include Tyk2, Jak1, Jak2, and Jak3. These kinases display significant sequence similarity and are generally catalytically inactive in resting cells.

[1462] The Jaks are activated by a wide range of receptors summarized in the Table below. (Adapted from review by Schidler and Darnell, Ann. Rev. Biochem. 64:621-51 (1995)). A cytokine receptor family, capable of activating Jaks, is divided into two groups: (a) Class 1 includes receptors for IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-11, IL-12, IL-15, Epo, PRL, GH, G-CSF, GM-CSF, LIF, CNTF, and thrombopoietin; and (b) Class 2 includes IFN-a, IFN-g, and IL-10. The Class 1 receptors share a conserved cysteine motif (a set of four conserved cysteines and one tryptophan) and a WSXWS motif (a membrane proximal region encoding Trp-Ser-Xaa-Trp-Ser (SEQ ID NO: 1113)).

[1463] Thus, on binding of a ligand to a receptor, Jaks are activated, which in turn

activate STATs, which then translocate and bind to GAS elements. This entire process is encompassed in the Jaks-STATs signal transduction pathway. Therefore, activation of the Jaks-STATs pathway, reflected by the binding of the GAS or the ISRE element, can be used to indicate proteins involved in the proliferation and differentiation of cells. For example, growth factors and cytokines are known to activate the Jaks-STATs pathway (See Table 5, below). Thus, by using GAS elements linked to reporter molecules, activators of the Jaks-STATs pathway can be identified.

Table 5

JAKs		STATS			GAS(elements) or ISRE	
Ligand	tyk2	Jak1	Jak2	Jak3		
IFN family						
IFN-a/B	+	+	-	-	1,2,3	ISRE
IFN-g		+	+	-	1	GAS (IRF1>Lys6>IFP)
Il-10	+	?	?	-	1,3	
gp130 family						
IL-6 (Pleiotropic)	+	+	+	?	1,3	GAS(IRF1>Lys6>IFP)
Il-11(Pleiotropic)	?	+	?	?	1,3	
OnM(Pleiotropic)	?	+	+	?	1,3	
LIF(Pleiotropic)	?	+	+	?	1,3	
CNTF(Pleiotropic)	-/+	+	+	?	1,3	
G-CSF(Pleiotropic)	?	+	?	?	1,3	
IL-12(Pleiotropic)	+	-	+	+	1,3	
g-C family						
IL-2 (lymphocytes)	-	+	-	+	1,3,5	GAS
IL-4 (lymph/myeloid)	-	+	-	+	6	GAS(IRF1=IFP>>Ly6)(IgH)
IL-7 (lymphocytes)	-	+	-	+	5	GAS
IL-9 (lymphocytes)	-	+	-	+	5	GAS
IL-13 (lymphocyte)	-	+	?	?	6	GAS
IL-15	?	+	?	+	5	GAS
gp140 family						
IL-3 (myeloid)	-	-	+	-	5	GAS(IRF1>IFP>>Ly6)
IL-5 (myeloid)	-	-	+	-	5	GAS
GM-CSF (myeloid)	-	-	+	-	5	GAS
Growth hormone family						
GH	?	-	+	-	5	
PRL	?	+/-	+	-	1,3,5	
EPO	?	-	+	-	5	GAS (B-CAS>IRF1=IFP>>Ly6)
Receptor Tyrosine Kinases						
EGF	?	+	+	-	1,3	GAS (IRF1)
PDGF	?	+	+	-	1,3	
CSF-1	?	+	+	-	1,3	GAS(not IRF1)

[1464] To construct a synthetic GAS containing promoter element, which is used in the Biological Assays described in Examples 78-80, a PCR based strategy is employed to generate a GAS-SV40 promoter sequence. The 5' primer contains four tandem copies of the GAS binding site found in the IRF1 promoter and previously demonstrated to bind STATs upon induction with a range of cytokines (Rothman et al., Immunity 1:457-468 (1994).), although other GAS or ISRE elements can be used instead. The 5' primer also contains 18bp of sequence complementary to the SV40 early promoter sequence and is flanked with an XhoI site. The sequence of the 5' primer is:

5':GCGCCTCGAGATTTCCCCGAAATCTAGATTTCCCCGAAATGATTTCCCCGAAA TGATTTCCCCGAAATATCTGCCATCTCAATTAG:3' (SEQ ID NO: 1114)

[1465] The downstream primer is complementary to the SV40 promoter and is flanked with a Hind III site: 5':GCGGCAAGCTTTTTGCAAAGCCTAGGC:3' (SEQ ID NO: 1115)

[1466] PCR amplification is performed using the SV40 promoter template present in the B-gal:promoter plasmid obtained from Clontech. The resulting PCR fragment is digested with XhoI/Hind III and subcloned into BLSK2-. (Stratagene.) Sequencing with forward and reverse primers confirms that the insert contains the following sequence:

5':CTCGAGATTTCCCCGAAATCTAGATTTCCCCGAAATGATTTCCCCGAAATGATT TCCCCGAAATATCTGCCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACT CCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCT GACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTC CAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTT:3' (SEQ ID NO:1116)

[1467] With this GAS promoter element linked to the SV40 promoter, a GAS:SEAP2 reporter construct is next engineered. Here, the reporter molecule is a secreted alkaline phosphatase, or "SEAP." Clearly, however, any reporter molecule can be instead of SEAP, in this or in any of the other Examples. Well known reporter molecules that can be used instead of SEAP include chloramphenicol acetyltransferase (CAT), luciferase, alkaline phosphatase, B-galactosidase, green fluorescent protein (GFP), or any protein detectable by an antibody.

[1468] The above sequence confirmed synthetic GAS-SV40 promoter element is subcloned into the pSEAP-Promoter vector obtained from Clontech using HindIII and XhoI, effectively replacing the SV40 promoter with the amplified GAS:SV40 promoter element, to

create the GAS-SEAP vector. However, this vector does not contain a neomycin resistance gene, and therefore, is not preferred for mammalian expression systems.

[1469] Thus, in order to generate mammalian stable cell lines expressing the GAS-SEAP reporter, the GAS-SEAP cassette is removed from the GAS-SEAP vector using SalI and NotI, and inserted into a backbone vector containing the neomycin resistance gene, such as pGFP-1 (Clontech), using these restriction sites in the multiple cloning site, to create the GAS-SEAP/Neo vector. Once this vector is transfected into mammalian cells, this vector can then be used as a reporter molecule for GAS binding as described in Examples 78-80.

[1470] Other constructs can be made using the above description and replacing GAS with a different promoter sequence. For example, construction of reporter molecules containing EGR and NF-KB promoter sequences are described in Examples 78-82. However, many other promoters can be substituted using the protocols described in these Examples. For instance, SRE, IL-2, NFAT, or Osteocalcin promoters can be substituted, alone or in combination (e.g., GAS/NF-KB/EGR, GAS/NF-KB, Il-2/NFAT, or NF-KB/GAS). Similarly, other cell lines can be used to test reporter construct activity, such as HELA (epithelial), HUVEC (endothelial), Reh (B-cell), Saos-2 (osteoblast), HUVAC (aortic), or Cardiomyocyte.

Example 76: Assay for SEAP Activity.

[1471] As a reporter molecule for the assays described in examples disclosed herein, SEAP activity is assayed using the Tropix Phospho-light Kit (Cat. BP-400) according to the following general procedure. The Tropix Phospho-light Kit supplies the Dilution, Assay, and Reaction Buffers used below.

[1472] Prime a dispenser with the 2.5x Dilution Buffer and dispense 15 ul of 2.5x dilution buffer into Optiplates containing 35 ul of a solution containing an albumin fusion protein of the invention. Seal the plates with a plastic sealer and incubate at 65 degree C for 30 min. Separate the Optiplates to avoid uneven heating.

[1473] Cool the samples to room temperature for 15 minutes. Empty the dispenser and prime with the Assay Buffer. Add 50 ml Assay Buffer and incubate at room temperature 5 min. Empty the dispenser and prime with the Reaction Buffer (see the Table below). Add 50 ul Reaction Buffer and incubate at room temperature for 20 minutes. Since the intensity of the chemiluminescent signal is time dependent, and it takes about 10 minutes to read 5

plates on a luminometer, thus one should treat 5 plates at each time and start the second set 10 minutes later.

[1474] Read the relative light unit in the luminometer. Set H12 as blank, and print the results. An increase in chemiluminescence indicates reporter activity.

Table 6

# of plates	Rxn buffer diluent (ml)	CSPD (ml)	# of plates	Rxn buffer diluent (ml)	CSPD (ml)
10	60	3	31	165	8.25
11	65	3.25	32	170	8.5
12	70	3.5	33	175	8.75
13	75	3.75	34	180	9
14	80	4	35	185	9.25
15	85	4.25	36	190	9.5
16	90	4.5	37	195	9.75
17	95	4.75	38	200	10
18	100	5	39	205	10.25
19	105	5.25	40	210	10.5
20	110	5.5	41	215	10.75
21	115	5.75	42	220	11
22	120	6	43	225	11.25
23	125	6.25	44	230	11.5
24	130	6.5	45	235	11.75
25	135	6.75	46	240	12
26	140	7	47	245	12.25
27	145	7.25	48	250	12.5
28	150	7.5	49	255	12.75
29	155	7.75	50	260	13
30	160	8			

Example 77: Assay Identifying Neuronal Activity.

[1475] When cells undergo differentiation and proliferation, a group of genes are activated through many different signal transduction pathways. One of these genes, EGR1 (early growth response gene 1), is induced in various tissues and cell types upon activation. The promoter of EGR1 is responsible for such induction. Using the EGR1 promoter linked to reporter molecules, the ability of fusion proteins of the invention to activate cells can be assessed.

[1476] Particularly, the following protocol is used to assess neuronal activity in PC12 cell lines. PC12 cells (rat phenochromocytoma cells) are known to proliferate and/or differentiate by activation with a number of mitogens, such as TPA (tetradecanoyl phorbol acetate), NGF (nerve growth factor), and EGF (epidermal growth factor). The EGR1 gene expression is activated during this treatment. Thus, by stably transfecting PC12 cells with a construct containing an EGR promoter linked to SEAP reporter, activation of PC12 cells by an albumin fusion protein of the present invention can be assessed.

[1477] The EGR/SEAP reporter construct can be assembled by the following protocol. The EGR-1 promoter sequence (-633 to +1)(Sakamoto K et al., Oncogene 6:867-871 (1991)) can be PCR amplified from human genomic DNA using the following primers:

First primer: 5' GCGCTCGAGGGATGACAGCGATAGAACCCCGG-3' (SEQ ID NO: 1117)

Second primer: 5' GCGAAGCTTCGCGACTCCCCGGATCCGCCTC-3' (SEQ ID NO: 1118)

[1478] Using the GAS:SEAP/Neo vector produced in Example 75, EGR1 amplified product can then be inserted into this vector. Linearize the GAS:SEAP/Neo vector using restriction enzymes XhoI/HindIII, removing the GAS/SV40 stuffer. Restrict the EGR1 amplified product with these same enzymes. Ligate the vector and the EGR1 promoter.

[1479] To prepare 96 well-plates for cell culture, two mls of a coating solution (1:30 dilution of collagen type I (Upstate Biotech Inc. Cat#08-115) in 30% ethanol (filter sterilized)) is added per one 10 cm plate or 50 ml per well of the 96-well plate, and allowed to air dry for 2 hr.

[1480] PC12 cells are routinely grown in RPMI-1640 medium (Bio Whittaker) containing 10% horse serum (JRH BIOSCIENCES, Cat. # 12449-78P), 5% heat-inactivated fetal bovine serum (FBS) supplemented with 100 units/ml penicillin and 100 ug/ml streptomycin on a precoated 10 cm tissue culture dish. One to four split is done every three

to four days. Cells are removed from the plates by scraping and resuspended with pipetting up and down for more than 15 times.

[1481] Transfect the EGR/SEAP/Neo construct into PC12 using techniques known in the art. EGR-SEAP/PC12 stable cells are obtained by growing the cells in 300 ug/ml G418. The G418-free medium is used for routine growth but every one to two months, the cells should be re-grown in 300 ug/ml G418 for couple of passages.

[1482] To assay for neuronal activity, a 10 cm plate with cells around 70 to 80% confluent is screened by removing the old medium. Wash the cells once with PBS (Phosphate buffered saline). Then starve the cells in low serum medium (RPMI-1640 containing 1% horse serum and 0.5% FBS with antibiotics) overnight.

[1483] The next morning, remove the medium and wash the cells with PBS. Scrape off the cells from the plate, suspend the cells well in 2 ml low serum medium. Count the cell number and add more low serum medium to reach final cell density as $5x10^5$ cells/ml.

[1484] Add 200 ul of the cell suspension to each well of 96-well plate (equivalent to $1x10^5$ cells/well). Add a series of different concentrations of an albumin fusion protein of the inventon, 37 degree C for 48 to 72 hr. As a positive control, a growth factor known to activate PC12 cells through EGR can be used, such as 50 ng/ul of Neuronal Growth Factor (NGF). Over fifty-fold induction of SEAP is typically seen in the positive control wells. SEAP assay may be routinely performed using techniques known in the art and/or as described in Example 76.

Example 78: Assay for T-cell Activity.

[1485] The following protocol is used to assess T-cell activity by identifying factors, and determining whether an albumin fusion protein of the invention proliferates and/or differentiates T-cells. T-cell activity is assessed using the GAS/SEAP/Neo construct produced in Example 75. Thus, factors that increase SEAP activity indicate the ability to activate the Jaks-STATS signal transduction pathway. The T-cell used in this assay is Jurkat T-cells (ATCC Accession No. TIB-152), although Molt-3 cells (ATCC Accession No. CRL-1552) and Molt-4 cells (ATCC Accession No. CRL-1582) cells can also be used.

[1486] Jurkat T-cells are lymphoblastic CD4+ Th1 helper cells. In order to generate stable cell lines, approximately 2 million Jurkat cells are transfected with the GAS-SEAP/neo

vector using DMRIE-C (Life Technologies)(transfection procedure described below). The transfected cells are seeded to a density of approximately 20,000 cells per well and transfectants resistant to 1 mg/ml genticin selected. Resistant colonies are expanded and then tested for their response to increasing concentrations of interferon gamma. The dose response of a selected clone is demonstrated.

[1487] Specifically, the following protocol will yield sufficient cells for 75 wells containing 200 ul of cells. Thus, it is either scaled up, or performed in multiple to generate sufficient cells for multiple 96 well plates. Jurkat cells are maintained in RPMI + 10% serum with 1%Pen-Strep. Combine 2.5 mls of OPTI-MEM (Life Technologies) with 10 ug of plasmid DNA in a T25 flask. Add 2.5 ml OPTI-MEM containing 50 ul of DMRIE-C and incubate at room temperature for 15-45 mins.

[1488] During the incubation period, count cell concentration, spin down the required number of cells (10^7 per transfection), and resuspend in OPTI-MEM to a final concentration of 10^7 cells/ml. Then add 1ml of 1 x 10^7 cells in OPTI-MEM to T25 flask and incubate at 37 degree C for 6 hrs. After the incubation, add 10 ml of RPMI + 15% serum.

[1489] The Jurkat:GAS-SEAP stable reporter lines are maintained in RPMI + 10% serum, 1 mg/ml Genticin, and 1% Pen-Strep. These cells are treated with varying concentrations of one or more fusion proteins of the present invention.

[1490] On the day of treatment with the fusion protein, the cells should be washed and resuspended in fresh RPMI + 10% serum to a density of 500,000 cells per ml. The exact number of cells required will depend on the number of fusion proteins and the number of different concentrations of fusion proteins being screened. For one 96 well plate, approximately 10 million cells (for 10 plates, 100 million cells) are required.

[1491] The well dishes containing Jurkat cells treated with the fusion protein are placed in an incubator for 48 hrs (note: this time is variable between 48-72 hrs). 35 ul samples from each well are then transferred to an opaque 96 well plate using a 12 channel pipette. The opaque plates should be covered (using sellophene covers) and stored at -20 degree C until SEAP assays are performed according to Example 76. The plates containing the remaining treated cells are placed at 4 degree C and serve as a source of material for repeating the assay on a specific well if desired.

[1492] As a positive control, 100 Unit/ml interferon gamma can be used which is known to activate Jurkat T cells. Over 30 fold induction is typically observed in the positive

control wells.

[1493] The above protocol may be used in the generation of both transient, as well as, stable transfected cells, which would be apparent to those of skill in the art.

Example 79: Assay for T-cell Activity.

[1494] NF-KB (Nuclear Factor KB) is a transcription factor activated by a wide variety of agents including the inflammatory cytokines IL-1 and TNF, CD30 and CD40, lymphotoxin-alpha and lymphotoxin-beta, by exposure to LPS or thrombin, and by expression of certain viral gene products. As a transcription factor, NF-KB regulates the expression of genes involved in immune cell activation, control of apoptosis (NF- KB appears to shield cells from apoptosis), B and T-cell development, anti-viral and antimicrobial responses, and multiple stress responses.

[1495] In non-stimulated conditions, NF- KB is retained in the cytoplasm with I-KB (Inhibitor KB). However, upon stimulation, I- KB is phosphorylated and degraded, causing NF- KB to shuttle to the nucleus, thereby activating transcription of target genes. Target genes activated by NF- KB include IL-2, IL-6, GM-CSF, ICAM-1 and class 1 MHC.

[1496] Due to its central role and ability to respond to a range of stimuli, reporter constructs utilizing the NF-KB promoter element are used to screen the fusion protein. Activators or inhibitors of NF-KB would be useful in treating, preventing, and/or diagnosing diseases. For example, inhibitors of NF-KB could be used to treat those diseases related to the acute or chronic activation of NF-KB, such as rheumatoid arthritis.

[1497] To construct a vector containing the NF-KB promoter element, a PCR based strategy is employed. The upstream primer contains four tandem copies of the NF-KB binding site (GGGGACTTTCCC) (SEQ ID NO: 1119), 18 bp of sequence complementary to the 5' end of the SV40 early promoter sequence, and is flanked with an XhoI site:

5':GCGGCCTCGAGGGGACTTTCCCGGGGACTTTCCGGGGACTTTCCGGGACTTTC CATCCTGCCATCTCAATTAG:3' (SEQ ID NO: 1120)

[1498] The downstream primer is complementary to the 3' end of the SV40 promoter and is flanked with a Hind III site:

5':GCGGCAAGCTTTTTGCAAAGCCTAGGC:3' (SEQ ID NO: 1115)

[1499] PCR amplification is performed using the SV40 promoter template present in the pB-gal:promoter plasmid obtained from Clontech. The resulting PCR fragment is

digested with XhoI and Hind III and subcloned into BLSK2-. (Stratagene) Sequencing with the T7 and T3 primers confirms the insert contains the following sequence:

5':CTCGAGGGGACTTTCCCGGGGACTTTCCGGGGACTTTCCGGGACTTTCCATCTG
CCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCC
CTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTAT
TTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGG
AGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTT:3' (SEQ ID NO: 1121)

[1500] Next, replace the SV40 minimal promoter element present in the pSEAP2-promoter plasmid (Clontech) with this NF-KB/SV40 fragment using XhoI and HindIII. However, this vector does not contain a neomycin resistance gene, and therefore, is not preferred for mammalian expression systems.

[1501] In order to generate stable mammalian cell lines, the NF-KB/SV40/SEAP cassette is removed from the above NF-KB/SEAP vector using restriction enzymes SalI and NotI, and inserted into a vector containing neomycin resistance. Particularly, the NF-KB/SV40/SEAP cassette was inserted into pGFP-1 (Clontech), replacing the GFP gene, after restricting pGFP-1 with SalI and NotI.

[1502] Once NF-KB/SV40/SEAP/Neo vector is created, stable Jurkat T-cells are created and maintained according to the protocol described in Example 76. Similarly, the method for assaying fusion proteins with these stable Jurkat T-cells is also described in Example 76. As a positive control, exogenous TNF alpha (0.1,1, 10 ng) is added to wells H9, H10, and H11, with a 5-10 fold activation typically observed.

Example 80: Assay Identifying Myeloid Activity.

[1503] The following protocol is used to assess myeloid activity of an albumin fusion protein of the present invention by determining whether the fusion protein proliferates and/or differentiates myeloid cells. Myeloid cell activity is assessed using the GAS/SEAP/Neo construct produced in Example 75. Thus, factors that increase SEAP activity indicate the ability to activate the Jaks-STATS signal transduction pathway. The myeloid cell used in this assay is U937, a pre-monocyte cell line, although TF-1, HL60, or KG1 can be used.

[1504] To transiently transfect U937 cells with the GAS/SEAP/Neo construct produced in Example 75, a DEAE-Dextran method (Kharbanda et. al., 1994, Cell Growth &

Differentiation, 5:259-265) is used. First, harvest $2x10^7$ U937 cells and wash with PBS. The U937 cells are usually grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin.

[1505] Next, suspend the cells in 1 ml of 20 mM Tris-HCl (pH 7.4) buffer containing 0.5 mg/ml DEAE-Dextran, 8 ug GAS-SEAP2 plasmid DNA, 140 mM NaCl, 5 mM KCl, 375 uM $Na_2HPO_4.7H_2O$, 1 mM $MgCl_2$, and 675 uM $CaCl_2$. Incubate at 37 degrees C for 45 min.

[1506] Wash the cells with RPMI 1640 medium containing 10% FBS and then resuspend in 10 ml complete medium and incubate at 37 degree C for 36 hr.

[1507] The GAS-SEAP/U937 stable cells are obtained by growing the cells in 400 ug/ml G418. The G418-free medium is used for routine growth but every one to two months, the cells should be re-grown in 400 ug/ml G418 for couple of passages.

[1508] These cells are tested by harvesting $1x10^8$ cells (this is enough for ten 96-well plates assay) and wash with PBS. Suspend the cells in 200 ml above described growth medium, with a final density of $5x10^5$ cells/ml. Plate 200 ul cells per well in the 96-well plate (or $1x10^5$ cells/well).

[1509] Add different concentrations of the fusion protein. Incubate at 37 degee C for 48 to 72 hr. As a positive control, 100 Unit/ml interferon gamma can be used which is known to activate U937 cells. Over 30 fold induction is typically observed in the positive control wells. SEAP assay the supernatant according to methods known in the art and/or the protocol described in Example 76.

Example 81: Assay Identifying Changes in Small Molecule Concentration and Membrane Permeability.

[1510] Binding of a ligand to a receptor is known to alter intracellular levels of small molecules, such as calcium, potassium, sodium, and pH, as well as alter membrane potential. These alterations can be measured in an assay to identify fusion proteins which bind to receptors of a particular cell. Although the following protocol describes an assay for calcium, this protocol can easily be modified to detect changes in potassium, sodium, pH, membrane potential, or any other small molecule which is detectable by a fluorescent probe.

[1511] The following assay uses Fluorometric Imaging Plate Reader ("FLIPR") to

measure changes in fluorescent molecules (Molecular Probes) that bind small molecules. Clearly, any fluorescent molecule detecting a small molecule can be used instead of the calcium fluorescent molecule, fluo-4 (Molecular Probes, Inc.; catalog no. F-14202), used here.

[1512] For adherent cells, seed the cells at 10,000 -20,000 cells/well in a Co-star black 96-well plate with clear bottom. The plate is incubated in a CO_2 incubator for 20 hours. The adherent cells are washed two times in Biotek washer with 200 ul of HBSS (Hank's Balanced Salt Solution) leaving 100 ul of buffer after the final wash.

[1513] A stock solution of 1 mg/ml fluo-4 is made in 10% pluronic acid DMSO. To load the cells with fluo-4, 50 ul of 12 ug/ml fluo-4 is added to each well. The plate is incubated at 37 degrees C in a CO_2 incubator for 60 min. The plate is washed four times in the Biotek washer with HBSS leaving 100 ul of buffer.

[1514] For non-adherent cells, the cells are spun down from culture media. Cells are re-suspended to $2\text{-}5x10^6$ cells/ml with HBSS in a 50-ml conical tube. 4 ul of 1 mg/ml fluo-4 solution in 10% pluronic acid DMSO is added to each ml of cell suspension. The tube is then placed in a 37 degrees C water bath for 30-60 min. The cells are washed twice with HBSS, resuspended to $1x10^6$ cells/ml, and dispensed into a microplate, 100 ul/well. The plate is centrifuged at 1000 rpm for 5 min. The plate is then washed once in Denley Cell Wash with 200 ul, followed by an aspiration step to 100 ul final volume.

[1515] For a non-cell based assay, each well contains a fluorescent molecule, such as fluo-4 . The fusion protein of the invention is added to the well, and a change in fluorescence is detected.

[1516] To measure the fluorescence of intracellular calcium, the FLIPR is set for the following parameters: (1) System gain is 300-800 mW; (2) Exposure time is 0.4 second; (3) Camera F/stop is F/2; (4) Excitation is 488 nm; (5) Emission is 530 nm; and (6) Sample addition is 50 ul. Increased emission at 530 nm indicates an extracellular signaling event caused by an albumin fusion protein of the present invention or a molecule induced by an albumin fusion protein of the present invention, which has resulted in an increase in the intracellular Ca^{++} concentration.

Example 82: Assay Identifying Tyrosine Kinase Activity.

[1517] The Protein Tyrosine Kinases (PTK) represent a diverse group of transmembrane and cytoplasmic kinases. Within the Receptor Protein Tyrosine Kinase (RPTK) group are receptors for a range of mitogenic and metabolic growth factors including the PDGF, FGF, EGF, NGF, HGF and Insulin receptor subfamilies. In addition there are a large family of RPTKs for which the corresponding ligand is unknown. Ligands for RPTKs include mainly secreted small proteins, but also membrane-bound and extracellular matrix proteins.

[1518] Activation of RPTK by ligands involves ligand-mediated receptor dimerization, resulting in transphosphorylation of the receptor subunits and activation of the cytoplasmic tyrosine kinases. The cytoplasmic tyrosine kinases include receptor associated tyrosine kinases of the src-family (e.g., src, yes, lck, lyn, fyn) and non-receptor linked and cytosolic protein tyrosine kinases, such as the Jak family, members of which mediate signal transduction triggered by the cytokine superfamily of receptors (e.g., the Interleukins, Interferons, GM-CSF, and Leptin).

[1519] Because of the wide range of known factors capable of stimulating tyrosine kinase activity, identifying whether an albumin fusion protein of the present invention or a molecule induced by a fusion proetin of the present invention is capable of activating tyrosine kinase signal transduction pathways is of interest. Therefore, the following protocol is designed to identify such molecules capable of activating the tyrosine kinase signal transduction pathways.

[1520] Seed target cells (e.g., primary keratinocytes) at a density of approximately 25,000 cells per well in a 96 well Loprodyne Silent Screen Plates purchased from Nalge Nunc (Naperville, IL). The plates are sterilized with two 30 minute rinses with 100% ethanol, rinsed with water and dried overnight. Some plates are coated for 2 hr with 100 ml of cell culture grade type I collagen (50 mg/ml), gelatin (2%) or polylysine (50 mg/ml), all of which can be purchased from Sigma Chemicals (St. Louis, MO) or 10% Matrigel purchased from Becton Dickinson (Bedford,MA), or calf serum, rinsed with PBS and stored at 4 degree C. Cell growth on these plates is assayed by seeding 5,000 cells/well in growth medium and indirect quantitation of cell number through use of alamarBlue as described by the manufacturer Alamar Biosciences, Inc. (Sacramento, CA) after 48 hr. Falcon plate covers #3071 from Becton Dickinson (Bedford,MA) are used to cover the Loprodyne Silent Screen Plates. Falcon Microtest III cell culture plates can also be used in some proliferation

experiments.

[1521] To prepare extracts, A431 cells are seeded onto the nylon membranes of Loprodyne plates (20,000/200ml/well) and cultured overnight in complete medium. Cells are quiesced by incubation in serum-free basal medium for 24 hr. After 5-20 minutes treatment with EGF (60ng/ml) or a different concentrations of an albumin fusion protein of the invention, the medium was removed and 100 ml of extraction buffer ((20 mM HEPES pH 7.5, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 2 mM Na3VO4, 2 mM Na4P2O7 and a cocktail of protease inhibitors (# 1836170) obtained from Boeheringer Mannheim (Indianapolis, IN)) is added to each well and the plate is shaken on a rotating shaker for 5 minutes at 4°C. The plate is then placed in a vacuum transfer manifold and the extract filtered through the 0.45 mm membrane bottoms of each well using house vacuum. Extracts are collected in a 96-well catch/assay plate in the bottom of the vacuum manifold and immediately placed on ice. To obtain extracts clarified by centrifugation, the content of each well, after detergent solubilization for 5 minutes, is removed and centrifuged for 15 minutes at 4 degree C at 16,000 x g.

[1522] Test the filtered extracts for levels of tyrosine kinase activity. Although many methods of detecting tyrosine kinase activity are known, one method is described here.

[1523] Generally, the tyrosine kinase activity of an albumin fusion protein of the invention is evaluated by determining its ability to phosphorylate a tyrosine residue on a specific substrate (a biotinylated peptide). Biotinylated peptides that can be used for this purpose include PSK1 (corresponding to amino acids 6-20 of the cell division kinase cdc2-p34) and PSK2 (corresponding to amino acids 1-17 of gastrin). Both peptides are substrates for a range of tyrosine kinases and are available from Boehringer Mannheim.

[1524] The tyrosine kinase reaction is set up by adding the following components in order. First, add 10ul of 5uM Biotinylated Peptide, then 10ul ATP/Mg_{2+} (5mM ATP/50mM $MgCl_2$), then 10ul of 5x Assay Buffer (40mM imidazole hydrochloride, pH7.3, 40 mM beta-glycerophosphate, 1mM EGTA, 100mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mg/ml BSA), then 5ul of Sodium Vanadate(1mM), and then 5ul of water. Mix the components gently and preincubate the reaction mix at 30 degree C for 2 min. Initial the reaction by adding 10ul of the control enzyme or the filtered supernatant.

[1525] The tyrosine kinase assay reaction is then terminated by adding 10 ul of

120mm EDTA and place the reactions on ice.

[1526] Tyrosine kinase activity is determined by transferring 50 ul aliquot of reaction mixture to a microtiter plate (MTP) module and incubating at 37 degree C for 20 min. This allows the streptavidin coated 96 well plate to associate with the biotinylated peptide. Wash the MTP module with 300ul/well of PBS four times. Next add 75 ul of anti-phospotyrosine antibody conjugated to horse radish peroxidase(anti-P-Tyr-POD(0.5u/ml)) to each well and incubate at 37 degree C for one hour. Wash the well as above.

[1527] Next add 100ul of peroxidase substrate solution (Boehringer Mannheim) and incubate at room temperature for at least 5 mins (up to 30 min). Measure the absorbance of the sample at 405 nm by using ELISA reader. The level of bound peroxidase activity is quantitated using an ELISA reader and reflects the level of tyrosine kinase activity.

Example 83: Assay Identifying Phosphorylation Activity.

[1528] As a potential alternative and/or complement to the assay of protein tyrosine kinase activity described in Example 82, an assay which detects activation (phosphorylation) of major intracellular signal transduction intermediates can also be used. For example, as described below one particular assay can detect tyrosine phosphorylation of the Erk-1 and Erk-2 kinases. However, phosphorylation of other molecules, such as Raf, JNK, p38 MAP, Map kinase kinase (MEK), MEK kinase, Src, Muscle specific kinase (MuSK), IRAK, Tec, and Janus, as well as any other phosphoserine, phosphotyrosine, or phosphothreonine molecule, can be detected by substituting these molecules for Erk-1 or Erk-2 in the following assay.

[1529] Specifically, assay plates are made by coating the wells of a 96-well ELISA plate with 0.1ml of protein G (1ug/ml) for 2 hr at room temp, (RT). The plates are then rinsed with PBS and blocked with 3% BSA/PBS for 1 hr at RT. The protein G plates are then treated with 2 commercial monoclonal antibodies (100ng/well) against Erk-1 and Erk-2 (1 hr at RT) (Santa Cruz Biotechnology). (To detect other molecules, this step can easily be modified by substituting a monoclonal antibody detecting any of the above described molecules.) After 3-5 rinses with PBS, the plates are stored at 4 degree C until use.

[1530] A431 cells are seeded at 20,000/well in a 96-well Loprodyne filterplate and cultured overnight in growth medium. The cells are then starved for 48 hr in basal medium (DMEM) and then treated with EGF (6ng/well) or varying concentrations of the fusion

protein of the invention for 5-20 minutes. The cells are then solubilized and extracts filtered directly into the assay plate.

[1531] After incubation with the extract for 1 hr at RT, the wells are again rinsed. As a positive control, a commercial preparation of MAP kinase (10ng/well) is used in place of A431 extract. Plates are then treated with a commercial polyclonal (rabbit) antibody (1ug/ml) which specifically recognizes the phosphorylated epitope of the Erk-1 and Erk-2 kinases (1 hr at RT). This antibody is biotinylated by standard procedures. The bound polyclonal antibody is then quantitated by successive incubations with Europium-streptavidin and Europium fluorescence enhancing reagent in the Wallac DELFIA instrument (time-resolved fluorescence). An increased fluorescent signal over background indicates a phosphorylation by the fusion protein of the present invention or a molecule induced by an albumin fusion protein of the present invention.

Example 84: Assay for the Stimulation of Bone Marrow CD34+ Cell Proliferation.

[1532] This assay is based on the ability of human CD34+ to proliferate in the presence of hematopoietic growth factors and evaluates the ability of fusion proteins of the inventon to stimulate proliferation of CD34+ cells.

[1533] It has been previously shown that most mature precursors will respond to only a single signal. More immature precursors require at least two signals to respond. Therefore, to test the effect of fusion proteins of the invention on hematopoietic activity of a wide range of progenitor cells, the assay contains a given fusion protein of the invention in the presence or absence of hematopoietic growth factors. Isolated cells are cultured for 5 days in the presence of Stem Cell Factor (SCF) in combination with tested sample. SCF alone has a very limited effect on the proliferation of bone marrow (BM) cells, acting in such conditions only as a "survival" factor. However, combined with any factor exhibiting stimulatory effect on these cells (e.g., IL-3), SCF will cause a synergistic effect. Therefore, if the tested fusion protein has a stimulatory effect on hematopoietic progenitors, such activity can be easily detected. Since normal BM cells have a low level of cycling cells, it is likely that any inhibitory effect of a given fusion protein might not be detected. Accordingly, assays for an inhibitory effect on progenitors is preferably tested in cells that are first subjected to *in vitro* stimulation with SCF+IL+3, and then contacted with the compound that is being evaluated for inhibition of such induced proliferation.

[1534] Briefly, CD34+ cells are isolated using methods known in the art. The cells are thawed and resuspended in medium (QBSF 60 serum-free medium with 1% L-glutamine (500ml) Quality Biological, Inc., Gaithersburg, MD Cat# 160-204-101). After several gentle centrifugation steps at 200 x g, cells are allowed to rest for one hour. The cell count is adjusted to 2.5 x 10^5 cells/ml. During this time, 100 µl of sterile water is added to the peripheral wells of a 96-well plate. The cytokines that can be tested with an albumin fusion protein of the invention in this assay is rhSCF (R&D Systems, Minneapolis, MN, Cat# 255-SC) at 50 ng/ml alone and in combination with rhSCF and rhIL-3 (R&D Systems, Minneapolis, MN, Cat# 203-ML) at 30 ng/ml. After one hour, 10 µl of prepared cytokines, varying concentrations of an albumin fusion protein of the invention, and 20 µl of diluted cells are added to the media which is already present in the wells to allow for a final total volume of 100 µl. The plates are then placed in a 37°C/5% CO_2 incubator for five days.

[1535] Eighteen hours before the assay is harvested, 0.5 µCi/well of [3H] Thymidine is added in a 10 µl volume to each well to determine the proliferation rate. The experiment is terminated by harvesting the cells from each 96-well plate to a filtermat using the Tomtec Harvester 96. After harvesting, the filtermats are dried, trimmed and placed into OmniFilter assemblies consisting of one OmniFilter plate and one OmniFilter Tray. 60 µl Microscint is added to each well and the plate sealed with TopSeal-A press-on sealing film A bar code 15 sticker is affixed to the first plate for counting. The sealed plates are then loaded and the level of radioactivity determined via the Packard Top Count and the printed data collected for analysis. The level of radioactivity reflects the amount of cell proliferation.

[1536] The studies described in this example test the activity of a given fusion protein to stimulate bone marrow CD34+ cell proliferation. One skilled in the art could easily modify the exemplified studies to test the activity of fusion porteins and polynucleotides of the invention (e.g., gene therapy) as well as agonists and antagonists thereof. The ability of an albumin fusion protein of the invention to stimulate the proliferation of bone marrow CD34+ cells indicates that the albumin fusion protein and/or polynucleotides corresponding to the fusion protein are useful for the diagnosis and treatment of disorders affecting the immune system and hematopoiesis. Representative uses are described in the "Immune Activity" and "Infectious Disease" sections above, and elsewhere herein.

Example 85: Assay for Extracellular Matrix Enhanced Cell Response (EMECR).

[1537] The objective of the Extracellular Matrix Enhanced Cell Response (EMECR) assay is to evaluate the ability of fusion proteins of the invention to act on hematopoietic stem cells in the context of the extracellular matrix (ECM) induced signal.

[1538] Cells respond to the regulatory factors in the context of signal(s) received from the surrounding microenvironment. For example, fibroblasts, and endothelial and epithelial stem cells fail to replicate in the absence of signals from the ECM. Hematopoietic stem cells can undergo self-renewal in the bone marrow, but not in *in vitro* suspension culture. The ability of stem cells to undergo self-renewal *in vitro* is dependent upon their interaction with the stromal cells and the ECM protein fibronectin (fn). Adhesion of cells to fn is mediated by the $\alpha_5.\beta_1$ and $\alpha_4.\beta_1$ integrin receptors, which are expressed by human and mouse hematopoietic stem cells. The factor(s) which integrate with the ECM environment and are responsible for stimulating stem cell self-renewal havea not yet been identified. Discovery of such factors should be of great interest in gene therapy and bone marrow transplant applications

[1539] Briefly, polystyrene, non tissue culture treated, 96-well plates are coated with fn fragment at a coating concentration of 0.2 $\mu g/ cm^2$. Mouse bone marrow cells are plated (1,000 cells/well) in 0.2 ml of serum-free medium. Cells cultured in the presence of IL-3 (5 ng/ml) + SCF (50 ng/ml) would serve as the positive control, conditions under which little self-renewal but pronounced differentiation of the stem cells is to be expected. Albumin fusion proteins of the invention are tested with appropriate negative controls in the presence and absence of SCF(5.0 ng/ml), where volume of the administed composition containing the albumin fusion protein of the invention represents 10% of the total assay volume. The plated cells are then allowed to grow by incubating in a low oxygen environment (5% CO_2, 7% O_2, and 88% N_2) tissue culture incubator for 7 days. The number of proliferating cells within the wells is then quantitated by measuring thymidine incorporation into cellular DNA. Verification of the positive hits in the assay will require phenotypic characterization of the cells, which can be accomplished by scaling up of the culture system and using appropriate antibody reagents against cell surface antigens and FACScan.

[1540] If a particular fusion protein of the present invention is found to be a stimulator of hematopoietic progenitors, the fusion protein and polynucleotides corresponding to the fusion protein may be useful for example, in the diagnosis and treatment

of disorders affecting the immune system and hematopoiesis. Representative uses are described in the "Immune Activity" and "Infectious Disease" sections above, and elsewhere herein. The fusion protein may also be useful in the expansion of stem cells and committed progenitors of various blood lineages, and in the differentiation and/or proliferation of various cell types.

[1541] Additionally, the albumin fusion proteins of the invention and polynucleotides encoding albumin fusion proteins of the invention, may also be employed to inhibit the proliferation and differentiation of hematopoietic cells and therefore may be employed to protect bone marrow stem cells from chemotherapeutic agents during chemotherapy. This antiproliferative effect may allow administration of higher doses of chemotherapeutic agents and, therefore, more effective chemotherapeutic treatment.

[1542] Moreover, fusion proteins of the invention and polynucleotides encoding albumin fusion proteins of the invention may also be useful for the treatment and diagnosis of hematopoietic related disorders such as, anemia, pancytopenia, leukopenia, thrombocytopenia or leukemia, since stromal cells are important in the production of cells of hematopoietic lineages. The uses include bone marrow cell ex-vivo culture, bone marrow transplantation, bone marrow reconstitution, radiotherapy or chemotherapy of neoplasia.

Example 86: Human Dermal Fibroblast and Aortic Smooth Muscle Cell Proliferation.

[1543] An albumin fusion protein of the invention is added to cultures of normal human dermal fibroblasts (NHDF) and human aortic smooth muscle cells (AoSMC) and two co-assays are performed with each sample. The first assay examines the effect of the fusion protein on the proliferation of normal human dermal fibroblasts (NHDF) or aortic smooth muscle cells (AoSMC). Aberrant growth of fibroblasts or smooth muscle cells is a part of several pathological processes, including fibrosis, and restenosis. The second assay examines IL6 production by both NHDF and SMC. IL6 production is an indication of functional activation. Activated cells will have increased production of a number of cytokines and other factors, which can result in a proinflammatory or immunomodulatory outcome. Assays are run with and without co-TNFa stimulation, in order to check for costimulatory or inhibitory activity.

[1544] Briefly, on day 1, 96-well black plates are set up with 1000 cells/well (NHDF) or 2000 cells/well (AoSMC) in 100 µl culture media. NHDF culture media contains:

Clonetics FB basal media, 1mg/ml hFGF, 5mg/ml insulin, 50mg/ml gentamycin, 2%FBS, while AoSMC culture media contains Clonetics SM basal media, 0.5 μg/ml hEGF, 5mg/ml insulin, 1μg/ml hFGF, 50mg/ml gentamycin, 50 μg/ml Amphotericin B, 5%FBS. After incubation at 37°C for at least 4-5 hours culture media is aspirated and replaced with growth arrest media. Growth arrest media for NHDF contains fibroblast basal media, 50mg/ml gentamycin, 2% FBS, while growth arrest media for AoSMC contains SM basal media, 50mg/ml gentamycin, 50μg/ml Amphotericin B, 0.4% FBS. Incubate at 37 °C until day 2.

[1545] On day 2, serial dilutions and templates of an albumin fusion protein of the invention are designed such that they always include media controls and known-protein controls. For both stimulation and inhibition experiments, proteins are diluted in growth arrest media. For inhibition experiments, TNFa is added to a final concentration of 2ng/ml (NHDF) or 5ng/ml (AoSMC). Add 1/3 vol media containing controls or an albumin fusion protein of the invention and incubate at 37 degrees C/5% CO_2 until day 5.

[1546] Transfer 60μl from each well to another labeled 96-well plate, cover with a plate-sealer, and store at 4 degrees C until Day 6 (for IL6 ELISA). To the remaining 100 μl in the cell culture plate, aseptically add Alamar Blue in an amount equal to 10% of the culture volume (10μl). Return plates to incubator for 3 to 4 hours. Then measure fluorescence with excitation at 530nm and emission at 590nm using the CytoFluor. This yields the growth stimulation/inhibition data.

[1547] On day 5, the IL6 ELISA is performed by coating a 96 well plate with 50-100 ul/well of Anti-Human IL6 Monoclonal antibody diluted in PBS, pH 7.4, incubate ON at room temperature.

[1548] On day 6, empty the plates into the sink and blot on paper towels. Prepare Assay Buffer containing PBS with 4% BSA. Block the plates with 200 μl/well of Pierce Super Block blocking buffer in PBS for 1-2 hr and then wash plates with wash buffer (PBS, 0.05% Tween-20). Blot plates on paper towels. Then add 50 μl/well of diluted Anti-Human IL-6 Monoclonal, Biotin-labeled antibody at 0.50 mg/ml. Make dilutions of IL-6 stock in media (30, 10, 3, 1, 0.3, 0 ng/ml). Add duplicate samples to top row of plate. Cover the plates and incubate for 2 hours at RT on shaker.

[1549] Plates are washed with wash buffer and blotted on paper towels. Dilute EU-labeled Streptavidin 1:1000 in Assay buffer, and add 100 μl/well. Cover the plate and

incubate 1 h at RT. Plates are again washed with wash buffer and blotted on paper towels.

[1550] Add 100 μl/well of Enhancement Solution. Shake for 5 minutes. Read the plate on the Wallac DELFIA Fluorometer. Readings from triplicate samples in each assay were tabulated and averaged.

[1551] A positive result in this assay suggests AoSMC cell proliferation and that the albumin fusion protein may be involved in dermal fibroblast proliferation and/or smooth muscle cell proliferation. A positive result also suggests many potential uses of the fusion protein and polynucleotides encoding the albumin fusion protein. For example, inflammation and immune responses, wound healing, and angiogenesis, as detailed throughout this specification. Particularly, fusion proteins may be used in wound healing and dermal regeneration, as well as the promotion of vasculogenesis, both of the blood vessels and lymphatics. The growth of vessels can be used in the treatment of, for example, cardiovascular diseases. Additionally, fusion proteins showing antagonistic activity in this assay may be useful in treating diseases, disorders, and/or conditions which involve angiogenesis by acting as an anti-vascular agent (e.g., anti-angiogenesis). These diseases, disorders, and/or conditions are known in the art and/or are described herein, such as, for example, malignancies, solid tumors, benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; artheroscleric plaques; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis, retinoblastoma, uvietis and Pterygia (abnormal blood vessel growth) of the eye; rheumatoid arthritis; psoriasis; delayed wound healing; endometriosis; vasculogenesis; granulations; hypertrophic scars (keloids); nonunion fractures; scleroderma; trachoma; vascular adhesions; myocardial angiogenesis; coronary collaterals; cerebral collaterals; arteriovenous malformations; ischemic limb angiogenesis; Osler-Webber Syndrome; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; fibromuscular dysplasia; wound granulation; Crohn's disease; and atherosclerosis. Moreover, albumin fusion proteins that act as antagonists in this assay may be useful in treating anti-hyperproliferative diseases and/or anti-inflammatory known in the art and/or described herein.

Example 87: Cellular Adhesion Molecule (CAM) Expression on Endothelial Cells.

[1552] The recruitment of lymphocytes to areas of inflammation and angiogenesis involves specific receptor-ligand interactions between cell surface adhesion molecules (CAMs) on lymphocytes and the vascular endothelium. The adhesion process, in both normal and pathological settings, follows a multi-step cascade that involves intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) expression on endothelial cells (EC). The expression of these molecules and others on the vascular endothelium determines the efficiency with which leukocytes may adhere to the local vasculature and extravasate into the local tissue during the development of an inflammatory response. The local concentration of cytokines and growth factor participate in the modulation of the expression of these CAMs.

[1553] Briefly, endothelial cells (e.g., Human Umbilical Vein Endothelial cells (HUVECs)) are grown in a standard 96 well plate to confluence, growth medium is removed from the cells and replaced with 100 μl of 199 Medium (10% fetal bovine serum (FBS)). Samples for testing (containing an albumin fusion protein of the invention) and positive or negative controls are added to the plate in triplicate (in 10 μl volumes). Plates are then incubated at 37°C for either 5 h (selectin and integrin expression) or 24 h (integrin expression only). Plates are aspirated to remove medium and 100 μl of 0.1% paraformaldehyde-PBS(with Ca++ and Mg++) is added to each well. Plates are held at 4°C for 30 min. Fixative is removed from the wells and wells are washed 1X with PBS(+Ca,Mg) + 0.5% BSA and drained. 10 μl of diluted primary antibody is added to the test and control wells. Anti-ICAM-1-Biotin, Anti-VCAM-1-Biotin and Anti-E-selectin-Biotin are used at a concentration of 10 μg/ml (1:10 dilution of 0.1 mg/ml stock antibody). Cells are incubated at 37°C for 30 min. in a humidified environment. Wells are washed three times with PBS(+Ca,Mg) + 0.5% BSA. 20 μl of diluted ExtrAvidin-Alkaline Phosphatase (1:5,000 dilution, referred to herein as the working dilution) are added to each well and incubated at 37°C for 30 min. Wells are washed three times with PBS(+Ca,Mg)+0.5% BSA. Dissolve 1 tablet of p-Nitrophenol Phosphate pNPP per 5 ml of glycine buffer (pH 10.4). 100 μl of pNPP substrate in glycine buffer is added to each test well. Standard wells in triplicate are prepared from the working dilution of the ExtrAvidin-Alkaline Phosphotase in glycine buffer: 1:5,000 (10^0) > $10^{-0.5}$ > 10^{-1} > $10^{-1.5}$. 5 μl of each dilution is added to triplicate wells and the resulting AP content in each well is 5.50 ng, 1.74 ng, 0.55 ng, 0.18 ng. 100 μl of pNNP reagent is then added to each of the standard wells. The plate is incubated at 37°C for 4h. A volume of 50 μl of 3M NaOH is

added to all wells. The plate is read on a plate reader at 405 nm using the background subtraction option on blank wells filled with glycine buffer only. Additionally, the template is set up to indicate the concentration of AP-conjugate in each standard well [5.50 ng; 1.74 ng; 0.55 ng; 0.18 ng]. Results are indicated as amount of bound AP-conjugate in each sample.

Example 88: Alamar Blue Endothelial Cells Proliferation Assay.

[1554] This assay may be used to quantitatively determine protein mediated inhibition of bFGF-induced proliferation of Bovine Lymphatic Endothelial Cells (LECs), Bovine Aortic Endothelial Cells (BAECs) or Human Microvascular Uterine Myometrial Cells (UTMECs). This assay incorporates a fluorometric growth indicator based on detection of metabolic activity. A standard Alamar Blue Proliferation Assay is prepared in EGM-2MV with 10 ng /ml of bFGF added as a source of endothelial cell stimulation. This assay may be used with a variety of endothelial cells with slight changes in growth medium and cell concentration. Dilutions of protein batches to be tested are diluted as appropriate. Serum-free medium (GIBCO SFM) without bFGF is used as a non-stimulated control and Angiostatin or TSP-1 are included as a known inhibitory controls.

[1555] Briefly, LEC, BAECs or UTMECs are seeded in growth media at a density of 5000 to 2000 cells/well in a 96 well plate and placed at 37 degreesC overnight. After the overnight incubation of the cells, the growth media is removed and replaced with GIBCO EC-SFM. The cells are treated with the appropriate dilutions of an albumin fusion protein of the invention or control protein sample(s) (prepared in SFM) in triplicate wells with additional bFGF to a concentration of 10 ng/ ml. Once the cells have been treated with the samples, the plate(s) is/are placed back in the 37° C incubator for three days. After three days 10 ml of stock alamar blue (Biosource Cat# DAL1100) is added to each well and the plate(s) is/are placed back in the 37°C incubator for four hours. The plate(s) are then read at 530nm excitation and 590nm emission using the CytoFluor fluorescence reader. Direct output is recorded in relative fluorescence units.

[1556] Alamar blue is an oxidation-reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. As cells grow in culture, innate metabolic activity results in a chemical reduction of the immediate surrounding environment. Reduction related to growth causes the indicator to

change from oxidized (non-fluorescent blue) form to reduced (fluorescent red) form (i.e., stimulated proliferation will produce a stronger signal and inhibited proliferation will produce a weaker signal and the total signal is proportional to the total number of cells as well as their metabolic activity). The background level of activity is observed with the starvation medium alone. This is compared to the output observed from the positive control samples (bFGF in growth medium) and protein dilutions.

Example 89: Detection of Inhibition of a Mixed Lymphocyte Reaction.

[1557] This assay can be used to detect and evaluate inhibition of a Mixed Lymphocyte Reaction (MLR) by fusion proteins of the invention. Inhibition of a MLR may be due to a direct effect on cell proliferation and viability, modulation of costimulatory molecules on interacting cells, modulation of adhesiveness between lymphocytes and accessory cells, or modulation of cytokine production by accessory cells. Multiple cells may be targeted by the albumin fusion proteins that inhibit MLR since the peripheral blood mononuclear fraction used in this assay includes T, B and natural killer lymphocytes, as well as monocytes and dendritic cells.

[1558] Albumin fusion proteins of the invention found to inhibit the MLR may find application in diseases associated with lymphocyte and monocyte activation or proliferation. These include, but are not limited to, diseases such as asthma, arthritis, diabetes, inflammatory skin conditions, psoriasis, eczema, systemic lupus erythematosus, multiple sclerosis, glomerulonephritis, inflammatory bowel disease, crohn's disease, ulcerative colitis, arteriosclerosis, cirrhosis, graft vs. host disease, host vs. graft disease, hepatitis, leukemia and lymphoma.

[1559] Briefly, PBMCs from human donors are purified by density gradient centrifugation using Lymphocyte Separation Medium (LSM®, density 1.0770 g/ml, Organon Teknika Corporation, West Chester, PA). PBMCs from two donors are adjusted to 2 x 10^6 cells/ml in RPMI-1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS and 2 mM glutamine. PBMCs from a third donor is adjusted to 2 x 10^5 cells/ml. Fifty microliters of PBMCs from each donor is added to wells of a 96-well round bottom microtiter plate. Dilutions of the fusion protein test material (50 µl) is added in triplicate to microtiter wells. Test samples (of the protein of interest) are added for final dilution of 1:4; rhuIL-2 (R&D Systems, Minneapolis, MN, catalog number 202-IL) is added to a final concentration

of 1 μg/ml; anti-CD4 mAb (R&D Systems, clone 34930.11, catalog number MAB379) is added to a final concentration of 10 μg/ml. Cells are cultured for 7-8 days at 37°C in 5% CO_2, and 1 μC of [^{3}H] thymidine is added to wells for the last 16 hrs of culture. Cells are harvested and thymidine incorporation determined using a Packard TopCount. Data is expressed as the mean and standard deviation of triplicate determinations.

[1560] Samples of the fusion protein of interest are screened in separate experiments and compared to the negative control treatment, anti-CD4 mAb, which inhibits proliferation of lymphocytes and the positive control treatment, IL-2 (either as recombinant material or supernatant), which enhances proliferation of lymphocytes.

Example 90: Assays for Protease Activity.

[1561] The following assay may be used to assess protease activity of an albumin fusion protein of the invention.

[1562] Gelatin and casein zymography are performed essentially as described (Heusen et al., *Anal. Biochem.*, 102:196-202 (1980); Wilson et al., *Journal of Urology*, 149:653-658 (1993)). Samples are run on 10% polyacryamide/0.1% SDS gels containing 1% gelain orcasein, soaked in 2.5% triton at room temperature for 1 hour, and in 0.1M glycine, pH 8.3 at 37°C 5 to 16 hours. After staining in amido black areas of proteolysis apear as clear areas agains the blue-black background. Trypsin (Sigma T8642) is used as a positive control.

[1563] Protease activity is also determined by monitoring the cleavage of n-a-benzoyl-L-arginine ethyl ester (BAEE) (Sigma B-4500. Reactions are set up in (25mM$NaPO_4$,1mM EDTA, and 1mM BAEE), pH 7.5. Samples are added and the change in adsorbance at 260nm is monitored on the Beckman DU-6 spectrophotometer in the time-drive mode. Trypsin is used as a positive control.

[1564] Additional assays based upon the release of acid-soluble peptides from casein or hemoglobin measured as adsorbance at 280 nm or colorimetrically using the Folin method are performed as described in Bergmeyer, et al., *Methods of Enzymatic Analysis*, 5 (1984). Other assays involve the solubilization of chromogenic substrates (Ward, *Applied Science*, 251-317 (1983)).

Example 91: Identifying Serine Protease Substrate Specificity.

[1565] Methods known in the art or described herein may be used to determine the substrate specificity of the albumin fusion proteins of the present invention having serine protease activity. A preferred method of determining substrate specificity is by the use of positional scanning synthetic combinatorial libraries as described in GB 2 324 529 (incorporated herein in its entirety).

Example 92: Ligand Binding Assays.

[1566] The following assay may be used to assess ligand binding activity of an albumin fusion protein of the invention.

[1567] Ligand binding assays provide a direct method for ascertaining receptor pharmacology and are adaptable to a high throughput format. The purified ligand for an albumin fusion protein of the invention is radiolabeled to high specific activity (50-2000 Ci/mmol) for binding studies. A determination is then made that the process of radiolabeling does not diminish the activity of the ligand towards the fusion protein. Assay conditions for buffers, ions, pH and other modulators such as nucleotides are optimized to establish a workable signal to noise ratio for both membrane and whole cell polypeptide sources. For these assays, specific polypeptide binding is defined as total associated radioactivity minus the radioactivity measured in the presence of an excess of unlabeled competing ligand. Where possible, more than one competing ligand is used to define residual nonspecific binding.

Example 93: Functional Assay in Xenopus Oocytes.

[1568] Capped RNA transcripts from linearized plasmid templates encoding an albumin fusion protein of the invention is synthesized in vitro with RNA polymerases in accordance with standard procedures. In vitro transcripts are suspended in water at a final concentration of 0.2 mg/mi. Ovarian lobes are removed from adult female toads, Stage V defolliculated oocytes are obtained, and RNA transcripts (10 ng/oocytc) are injected in a 50 nl bolus using a microinjection apparatus. Two electrode voltage clamps are used to measure the currents from individual *Xenopus oocytes* in response fusion protein and polypeptide agonist exposure. Recordings are made in Ca2+ free Barth's medium at room temperature. The Xenopus system can be used to screen known ligands and tissue/cell extracts for activating ligands.

Example 94: Microphysiometric Assays.

[1569] Activation of a wide variety of secondary messenger systems results in extrusion of small amounts of acid from a cell. The acid formed is largely as a result of the increased metabolic activity required to fuel the intracellular signaling process. The pH changes in the media surrounding the cell are very small but are detectable by the CYTOSENSOR microphysiometer (Molecular Devices Ltd., Menlo Park, Calif.). The CYTOSENSOR is thus capable of detecting the ability of an albumin fusion protein of the invention to activate secondary messengers that are coupled to an energy utilizing intracellular signaling pathway.

Example 95: Extract/Cell Supernatant Screening.

[1570] A large number of mammalian receptors exist for which there remains, as yet, no cognate activating ligand (agonist). Thus, active ligands for these receptors may not be included within the ligands banks as identified to date. Accordingly, the albumin fusion proteins of the invention can also be functionally screened (using calcium, cAMP, microphysiometer, oocyte electrophysiology, etc., functional screens) against tissue extracts to identify natural ligands for the Therapeutic protein portion and/or albumin protein portion of an albumin fusion protein of the invention. Extracts that produce positive functional responses can be sequentially subfractionated until an activating ligand is isolated and identified.

Example 96: ATP-binding assay.

[1571] The following assay may be used to assess ATP-binding activity of fusion proteins of the invention.

[1572] ATP-binding activity of an albumin fusion protein of the invention may be detected using the ATP-binding assay described in U.S. Patent 5,858,719, which is herein incorporated by reference in its entirety. Briefly, ATP-binding to an albumin fusion protein of the invention is measured via photoaffinity labeling with 8-azido-ATP in a competition assay. Reaction mixtures containing 1 mg/ml of ABC transport protein are incubated with varying concentrations of ATP, or the non-hydrolyzable ATP analog adenyl-5'-imidodiphosphate for 10 minutes at 4°C. A mixture of 8-azido-ATP (Sigma Chem. Corp., St. Louis, MO.) plus 8-

azido-ATP (^{32}P-ATP) (5 mCi/μmol, ICN, Irvine CA.) is added to a final concentration of 100 μM and 0.5 ml aliquots are placed in the wells of a porcelain spot plate on ice. The plate is irradiated using a short wave 254 nm UV lamp at a distance of 2.5 cm from the plate for two one-minute intervals with a one-minute cooling interval in between. The reaction is stopped by addition of dithiothreitol to a final concentration of 2mM. The incubations are subjected to SDS-PAGE electrophoresis, dried, and autoradiographed. Protein bands corresponding to the albumin fusion proteins of the invention are excised, and the radioactivity quantified. A decrease in radioactivity with increasing ATP or adenly-5'-imidodiphosphate provides a measure of ATP affinity to the fusion protein.

Example 97: Phosphorylation Assay.

[1573] In order to assay for phosphorylation activity of an albumin fusion protein of the invention, a phosphorylation assay as described in U.S. Patent 5,958,405 (which is herein incorporated by reference) is utilized. Briefly, phosphorylation activity may be measured by phosphorylation of a protein substrate using gamma-labeled ^{32}P-ATP and quantitation of the incorporated radioactivity using a gamma radioisotope counter. The fusion portein of the invention is incubated with the protein substrate, ^{32}P-ATP, and a kinase buffer. The ^{32}P incorporated into the substrate is then separated from free ^{32}P-ATP by electrophoresis, and the incorporated ^{32}P is counted and compared to a negative control. Radioactivity counts above the negative control are indicative of phosphorylation activity of the fusion protein.

Example 98: Detection of Phosphorylation Activity (Activation) of an Albumin Fusion Protein of the Invention in the Presence of Polypeptide Ligands.

[1574] Methods known in the art or described herein may be used to determine the phosphorylation activity of an albumin fusion protein of the invention. A preferred method of determining phosphorylation activity is by the use of the tyrosine phosphorylation assay as described in US 5,817,471 (incorporated herein by reference).

Example 99: Identification Of Signal Transduction Proteins That Interact With An albumin fusion protein Of The Present Invention.

[1575] Albumin fusion proteins of the invention may serve as research tools for the identification, characterization and purification of signal transduction pathway proteins or

receptor proteins. Briefly, a labeled fusion protein of the invention is useful as a reagent for the purification of molecules with which it interacts. In one embodiment of affinity purification, an albumin fusion protein of the invention is covalently coupled to a chromatography column. Cell-free extract derived from putative target cells, such as carcinoma tissues, is passed over the column, and molecules with appropriate affinity bind to the albumin fusion protein. The protein complex is recovered from the column, dissociated, and the recovered molecule subjected to N-terminal protein sequencing. This amino acid sequence is then used to identify the captured molecule or to design degenerate oligonucleotide probes for cloning the relevant gene from an appropriate cDNA library.

Example 100: IL-6 Bioassay.

[1576] A variety of assays are known in the art for testing the proliferative effects of an albumin fusion protein of the invention. For example, one such asssay is the IL-6 Bioassay as described by Marz *et al.* (*Proc. Natl. Acad. Sci., U.S.A., 95*:3251-56 (1998), which is herein incorporated by reference). After 68 hrs. at 37°C, the number of viable cells is measured by adding the tetrazolium salt thiazolyl blue (MTT) and incubating for a further 4 hrs. at 37°C. B9 cells are lysed by SDS and optical density is measured at 570 nm. Controls containing IL-6 (positive) and no cytokine (negative) are Briefly, IL-6 dependent B9 murine cells are washed three times in IL-6 free medium and plated at a concentration of 5,000 cells per well in 50 μl, and 50 μl of fusion protein of the invention is added. utilized. Enhanced proliferation in the test sample(s) (containing an albumin fusion protein of the invention) relative to the negative control is indicative of proliferative effects mediated by the fusion protein.

Example 101: Support of Chicken Embryo Neuron Survival.

[1577] To test whether sympathetic neuronal cell viability is supported by an albumin fusion protein of the invention, the chicken embryo neuronal survival assay of Senaldi *et al* may be utilized (*Proc. Natl. Acad. Sci., U.S.A., 96*:11458-63 (1998), which is herein incorporated by reference). Briefly, motor and sympathetic neurons are isolated from chicken embryos, resuspended in L15 medium (with 10% FCS, glucose, sodium selenite, progesterone, conalbumin, putrescine, and insulin; Life Technologies, Rockville, MD.) and Dulbecco's modified Eagles medium [with 10% FCS, glutamine, penicillin, and 25 mM

Hepes buffer (pH 7.2); Life Technologies, Rockville, MD.], respectively, and incubated at 37°C in 5% CO_2 in the presence of different concentrations of the purified fusion protein of the invention, as well as a negative control lacking any cytokine. After 3 days, neuron survival is determined by evaluation of cellular morphology, and through the use of the colorimetric assay of Mosmann (Mosmann, T., *J. Immunol. Methods, 65*:55-63 (1983)). Enhanced neuronal cell viability as compared to the controls lacking cytokine is indicative of the ability of the albumin fusion protein to enhance the survival of neuronal cells.

Example 102: Assay for Phosphatase Activity.

[1578] The following assay may be used to assess serine/threonine phosphatase (PTPase) activity of an albumin fusion protein of the invention.

[1579] In order to assay for serine/threonine phosphatase (PTPase) activity, assays can be utilized which are widely known to those skilled in the art. For example, the serine/threonine phosphatase (PSPase) activity of an albumin fusion protein of the invention may be measured using a PSPase assay kit from New England Biolabs, Inc. Myelin basic protein (MyBP), a substrate for PSPase, is phosphorylated on serine and threonine residues with cAMP-dependent Protein Kinase in the presence of [^{32}P]ATP. Protein serine/threonine phosphatase activity is then determined by measuring the release of inorganic phosphate from 32P-labeled MyBP.

Example 103: Interaction of Serine/Threonine Phosphatases with other Proteins.

[1580] Fusion protein of the invention having serine/threonine phosphatase activity (e.g., as determined in Example 102) are useful, for example, as research tools for the identification, characterization and purification of additional interacting proteins or receptor proteins, or other signal transduction pathway proteins. Briefly, a labeled fusion protein of the invention is useful as a reagent for the purification of molecules with which it interacts. In one embodiment of affinity purification, an albumin fusion protein of the invention is covalently coupled to a chromatography column. Cell-free extract derived from putative target cells, such as neural or liver cells, is passed over the column, and molecules with appropriate affinity bind to the fusion protein. The fusion protein -complex is recovered from the column, dissociated, and the recovered molecule subjected to N-terminal protein sequencing. This amino acid sequence is then used to identify the captured molecule or to

design degenerate oligonucleotide probes for cloning the relevant gene from an appropriate cDNA library.

Example 104: Assaying for Heparanase Activity.

[1581] There a numerous assays known in the art that may be employed to assay for heparanase activity of an albumin fusion protein of the invention. In one example, heparanase activity of an albumin fusion protein of the invention, is assayed as described by Vlodavsky et al., (Vlodavsky et al., Nat. Med., 5:793-802 (1999)). Briefly, cell lysates, conditioned media, intact cells (1 x 10^6 cells per 35-mm dish), cell culture supernatant, or purified fusion protein are incubated for 18 hrs at 37°C, pH 6.2-6.6, with ^{35}S-labeled ECM or soluble ECM derived peak I proteoglycans. The incubation medium is centrifuged and the supernatant is analyzed by gel filtration on a Sepharose CL-6B column (0.9 x 30 cm). Fractions are eluted with PBS and their radioactivity is measured. Degradation fragments of heparan sulfate side chains are eluted from Sepharose 6B at $0.5 < K_{av} < 0.8$ (peak II). Each experiment is done at least three times. Degradation fragments corresponding to "peak II," as described by Vlodavsky et al., is indicative of the activity of an albumin fusion protein of the invention in cleaving heparan sulfate.

Example 105: Immobilization of biomolecules.

[1582] This example provides a method for the stabilization of an albumin fusion protein of the invention in non-host cell lipid bilayer constucts (see, e.g., Bieri et al., Nature Biotech 17:1105-1108 (1999), hereby incorporated by reference in its entirety herein) which can be adapted for the study of fusion proteins of the invention in the various functional assays described above. Briefly, carbohydrate-specific chemistry for biotinylation is used to confine a biotin tag to an albumin fusion protein of the invention, thus allowing uniform orientation upon immobilization. A 50uM solution of an albumin fusion protein of the invention in washed membranes is incubated with 20 mM NaIO4 and 1.5 mg/ml (4mM) BACH or 2 mg/ml (7.5mM) biotin-hydrazide for 1 hr at room temperature (reaction volume, 150ul). Then the sample is dialyzed (Pierce Slidealizer Cassett, 10 kDa cutoff; Pierce Chemical Co., Rockford IL) at 4C first for 5 h, exchanging the buffer after each hour, and finally for 12 h against 500 ml buffer R (0.15 M NaCl, 1 mM MgCl2, 10 mM sodium phosphate, pH7). Just before addition into a cuvette, the sample is diluted 1:5 in buffer

ROG50 (Buffer R supplemented with 50 mM octylglucoside).

Example 106: Assays for Metalloproteinase Activity.

[1583] Metalloproteinases are peptide hydrolases which use metal ions, such as Zn^{2+}, as the catalytic mechanism. Metalloproteinase activity of an albumin fusion protein of the present invention can be assayed according to methods known in the art. The following exemplary methods are provided:

Proteolysis of alpha-2-macroglobulin

[1584] To confirm protease activity, a purified fusion protein of the invention is mixed with the substrate alpha-2-macroglobulin (0.2 unit/ml; Boehringer Mannheim, Germany) in 1x assay buffer (50 mM HEPES, pH 7.5, 0.2 M NaCl, 10 mM $CaCl_2$, 25 μM $ZnCl_2$ and 0.05% Brij-35) and incubated at 37°C for 1-5 days. Trypsin is used as positive control. Negative controls contain only alpha-2-macroglobulin in assay buffer. The samples are collected and boiled in SDS-PAGE sample buffer containing 5% 2-mercaptoethanol for 5-min, then loaded onto 8% SDS-polyacrylamide gel. After electrophoresis the proteins are visualized by silver staining. Proteolysis is evident by the appearance of lower molecular weight bands as compared to the negative control.

Inhibition of alpha-2-macroglobulin proteolysis by inhibitors of metalloproteinases

[1585] Known metalloproteinase inhibitors (metal chelators (EDTA, EGTA, AND $HgCl_2$), peptide metalloproteinase inhibitors (TIMP-1 and TIMP–2), and commercial small molecule MMP inhibitors) may also be used to characterize the proteolytic activity of an albumin fusion protein of the invention. Three synthetic MMP inhibitors that may be used are: MMP inhibitor I, [IC_{50} = 1.0 μM against MMP-1 and MMP–8; IC_{50} = 30 μM against MMP-9; IC_{50} = 150 μM against MMP-3]; MMP-3 (stromelysin-1) inhibitor I [IC_{50} = 5 μM against MMP-3], and MMP-3 inhibitor II [K_i = 130 nM against MMP-3]; inhibitors available through Calbiochem, catalog # 444250, 444218, and 444225, respectively). Briefly, different concentrations of the small molecule MMP inhibitors are mixed with a purified fusion protein of the invention (50μg/ml) in 22.9 μl of 1x HEPES buffer (50 mM HEPES, pH 7.5, 0.2 M NaCl, 10 mM $CaCl_2$, 25 μM $ZnCl_2$ and 0.05%Brij-35) and incubated at room temperature (24 °C) for 2-hr, then 7.1 μl of substrate alpha-2-macroglobulin (0.2 unit/ml) is added and

incubated at 37°C for 20-hr. The reactions are stopped by adding 4x sample buffer and boiled immediately for 5 minutes. After SDS-PAGE, the protein bands are visualized by silver stain.

Synthetic Fluorogenic Peptide Substrates Cleavage Assay

[1586] The substrate specificity for fusion proteins of the invention with demonstrated metalloproteinase activity may be determined using techniques knonw in the art, such as using synthetic fluorogenic peptide substrates (purchased from BACHEM Bioscience Inc). Test substrates include, M-1985, M-2225, M-2105, M-2110, and M-2255. The first four are MMP substrates and the last one is a substrate of tumor necrosis factor-α (TNF-α) converting enzyme (TACE). These substrastes are preferably prepared in 1:1 dimethyl sulfoxide (DMSO) and water. The stock solutions are 50-500 μM. Fluorescent assays are performed by using a Perkin Elmer LS 50B luminescence spectrometer equipped with a constant temperature water bath. The excitation λ is 328 nm and the emission λ is 393 nm. Briefly, the assay is carried out by incubating 176 μl 1x HEPES buffer (0.2 M NaCl, 10 mM $CaCl_2$, 0.05% Brij-35 and 50 mM HEPES, pH 7.5) with 4 μl of substrate solution (50 μM) at 25 °C for 15 minutes, and then adding 20 μl of a purified fusion protein of the invention into the assay cuvett. The final concentration of substrate is 1 μM. Initial hydrolysis rates are monitored for 30-min.

Example 107:Identification and Cloning of VH and VL domains.

[1587] One method to identfy and clone VH and VL domains from cell lines expressing a particular antibody is to perform PCR with VH and VL specific primers on cDNA made from the antibody expressing cell lines. Briefly, RNA is isolated from the cell lines and used as a template for RT-PCR designed to amplify the VH and VL domains of the antibodies expressed by the EBV cell lines. Cells may be lysed in the TRIzol® reagent (Life Technologies, Rockville. MD) and extracted with one fifth volume of chloroform. After addition of chloroform, the solution is allowed to incubate at room temperature for 10 minutes, and the centrifuged at 14,000 rpm for 15 minutes at 4°C in a tabletop centrifuge. The supernatant is collected and RNA is precipitated using an equal volume of isopropanol. Precipitated RNA is pelleted by centrifuging at 14,000 rpm for 15 minutes at 4°C in a tabletop centrifuge. Following centrifugation, the supernatant is discarded and washed with

75% ethanol. Follwing washing, the RNA is centrifuged again at 800 rpm for 5 minutes at 4°C. The supernatant is discarded and the pellet allowed to air dry. RNA is the dissolved in DEPC water and heated to 60°C for 10 minutes. Quantities of RNA can determined using optical density measurements.

cDNA may be synthesized, according to methods well-known in the art, from 1.5-2.5 micrograms of RNA using reverse transciptase and random hexamer primers. cDNA is then used as a template for PCR amplification of VH and VL domains. Primers used to amplify VH and VL genes are shown in Table 7. Typically a PCR reaction makes use of a single 5' primer and a single 3' primer. Sometimes, when the amount of available RNA template is limiting, or for greater efficiency, groups of 5' and/or 3' primers may be used. For example, sometimes all five VH-5' primers and all JH3' primers are used in a single PCR reaction. The PCR reaction is carried out in a 50 microliter volume containing 1X PCR buffer, 2mM of each dNTP, 0.7 units of High Fidelity Taq polymerse, 5' primer mix, 3' primer mix and 7.5 microliters of cDNA. The 5' and 3' primer mix of both VH and VL can be made by pooling together 22 pmole and 28 pmole, respectively, of each of the individual primers. PCR conditions are: 96°C for 5 minutes; followed by 25 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute; followed by an extension cycle of 72°C for 10 minutes. After the reaction is completed, sample tubes are stored 4°C.

Table 7: Primer Sequences Used to Amplify VH and VL domains.

Primer name	SEQ ID NO	Primer Sequence (5'-3')
VH Primers		
Hu VH1-5'	1056	CAGGTGCAGCTGGTGCAGTCTGG
Hu VH2-5'	1057	CAGGTCAACTTAAGGGAGTCTGG
Hu VH3-5'	1058	GAGGTGCAGCTGGTGGAGTCTGG
Hu VH4-5'	1059	CAGGTGCAGCTGCAGGAGTCGGG
Hu VH5-5'	1060	GAGGTGCAGCTGTTGCAGTCTGC
Hu VH6-5'	1061	CAGGTACAGCTGCAGCAGTCAGG
Hu JH1,2-5'	1062	TGAGGAGACGGTGACCAGGGTGCC
Hu JH3-5'	1063	TGAAGAGACGGTGACCATTGTCCC
Hu JH4,5-5'	1064	TGAGGAGACGGTGACCAGGGTTCC
Hu JH6-5'	1065	TGAGGAGACGGTGACCGTGGTCCC
VL Primers		
Hu Vkappa1-5'	1066	GACATCCAGATGACCCAGTCTCC
Hu Vkappa2a-5'	1067	GATGTTGTGATGACTCAGTCTCC
Hu Vkappa2b-5'	1068	GATATTGTGATGACTCAGTCTCC
Hu Vkappa3-5'	1069	GAAATTGTGTTGACGCAGTCTCC
Hu Vkappa4-5'	1070	GACATCGTGATGACCCAGTCTCC
Hu Vkappa5-5'	1071	GAAACGACACTCACGCAGTCTCC
Hu Vkappa6-5'	1072	GAAATTGTGCTGACTCAGTCTCC
Hu Vlambda1-5'	1073	CAGTCTGTGTTGACGCAGCCGCC
Hu Vlambda2-5'	1074	CAGTCTGCCCTGACTCAGCCTGC
Hu Vlambda3-5'	1075	TCCTATGTGCTGACTCAGCCACC
Hu Vlambda3b-5'	1076	TCTTCTGAGCTGACTCAGGACCC
Hu Vlambda4-5'	1077	CACGTTATACTGACTCAACCGCC
Hu Vlambda5-5'	1078	CAGGCTGTGCTCACTCAGCCGTC
Hu Vlambda6-5'	1079	AATTTTATGCTGACTCAGCCCCA
Hu Jkappa1-3'	1080	ACGTTTGATTTCCACCTTGGTCCC
Hu Jkappa2-3'	1081	ACGTTTGATCTCCAGCTTGGTCCC
Hu Jkappa3-3'	1082	ACGTTTGATATCCACTTTGGTCCC
Hu Jkappa4-3'	1083	ACGTTTGATCTCCACCTTGGTCCC
Hu Jkappa5-3'	1084	ACGTTTAATCTCCAGTCGTGTCCC
Hu Jlambda1-3'	1085	CAGTCTGTGTTGACGCAGCCGCC
Hu Jlambda2-3'	1086	CAGTCTGCCCTGACTCAGCCTGC
Hu Jlambda3--3'	1087	TCCTATGTGCTGACTCAGCCACC
Hu Jlambda3b-3'	1088	TCTTCTGAGCTGACTCAGGACCC
Hu Jlambda4-3'	1089	CACGTTATACTGACTCAACCGCC
Hu Jlambda5-3'	1090	CAGGCTGTGCTCACTCAGCCGTC
Hu Jlambda6-3'	1091	AATTTTATGCTGACTCAGCCCCA

PCR samples are then electrophoresed on a 1.3% agarose gel. DNA bands of the expected sizes (~506 base pairs for VH domains, and 344 base pairs for VL domains) can be cut out of the gel and purified using methods well known in the art. Purified PCR products can be ligated into a PCR cloning vector (TA vector from Invitrogen Inc., Carlsbad, CA). Individual cloned PCR products can be isolated after transfection of E. coli and blue/white color selection. Cloned PCR products may then be sequenced using methods commonly known in the art.

[1588] The PCR bands containing the VH domain and the VL domains can also be used to create full-length Ig expression vectors. VH and VL domains can be cloned into vectors containing the nucleotide sequences of a heavy (e.g., human IgG1 or human IgG4) or light chain (human kappa or human lambda) constant regions such that a complete heavy or light chain molecule could be expressed from these vectors when transfected into an appropriate host cell. Further, when cloned heavy and light chains are both expressed in one cell line (from either one or two vectors), they can assemble into a complete functional antibody molecule that is secreted into the cell culture medium. Methods using polynucleotides encoding VH and VL antibody domain to generate expression vectors that encode complete antibody molecules are well known within the art.

EXAMPLE 108: Construct ID 2672, HSA-T20, Generation.

[1589] Construct ID 2672 (SEQ ID NO:1186), pSAC35:HSA.T20, comprises DNA encoding a T20 albumin fusion protein which has full length HSA fused to the amino-terminus of the HIV-1 inhibitory peptide T20, i.e., Y643-F678, in the yeast *S. cerevisiae* expression vector pSAC35. The T20 peptide is derived from the ectodomain of the HIV-1 transmembrane protein gp41 and is shown to have inhibitory activity on HIV-1 infection.

Cloning of T20 cDNA

[1590] The polynucleotide encoding T20 was PCR generated using four overlapping primers T20-1, T20-2, T20-3, and T20-4, described below. The sequence was codon optimized for expression in yeast *S. cerevisiae*. The PCR fragment was cut with *Bsu* 36I/*Asc* I, and ligated into *Bsu* 36I/*Asc* I cut pScNHSA. A *Not* I fragment was then subcloned into the pSAC35 plasmid. Construct ID #2672 encodes an albumin fusion protein containing full length HSA and the HIV-1 inhibitory peptide T20, i.e., Tyr-643 to Phe-678 (SEQ ID

NO:1188).

[1591] The 5' and 3' primers of the four overlapping oligonucleotides suitable for PCR amplification of the polynucleotide encoding the HIV-1 inhibitory peptide T20, T20-1 and T20-4, were synthesized:

T20-1: 5'-AAGCTGCCTTAGGCTTA**TACACTAGTTTGATTCATAGTTTG**-3' (SEQ ID NO:1189)

T20-2: 5'-**TACACTAGTTTGATTCATAGTTTGATTGAAGAAAGTCAAAATCAACAAGAAAA GAATGAACAAG**-3' (SEQ ID NO:1204)

T20-3: 5'-**AAACCAATTCCACAAACTAGCCCATTTATCCAATTCCAACAATTCTTGTTCATT CTTTTCTTGTTGAT**-3' (SEQ ID NO:1205)

T20-4: 5'-TTGGCGCGCC**TTAAAACCAATTCCACAAACTAGCCCATTTATCC**-3' (SEQ ID NO:1190)

[1592] T20-1 incorporates the *Bsu* 36I cloning site (shown underlined) and nucleotides encoding the last four amino acid residues of the mature form of HSA (SEQ ID NO:1038), as well as 24 nucleotides (shown in bold) encoding the first 8 amino acid residues of the HIV-1 inhibitory peptide T20, i.e., Tyr-643 to Leu-650. In T20-4, the *Asc* I site is underlined and the last 31 nucleotides (shown in bold) are the reverse complement of DNA encoding the last 10 amino acid residues of the HIV-1 inhibitory peptide T20, Asp-669 to Phe-678. The T20-2 and T20-3 oligonucleotides overlap with each other and with T20-1 and T20-4, respectively, and encode the HIV-1 inhibitory peptide T20. The PCR product was purified (for example, using Wizard PCR Preps DNA Purification System (Promega Corp)) and then digested with *Bsu36*I and *AscI*. After further purification of the *Bsu36*I-*AscI* fragment by gel electrophoresis, the product was cloned into *Bsu36I*/*AscI* digested pScNHSA. After the sequence was confirmed, the expression cassette encoding this T20 albumin fusion protein was subcloned into pSAC35 as a *Not* I fragment. A *Not* I fragment was further subcloned into pSAC35 to give construct ID # 2672.

[1593] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing can confirm the presence of the expected HSA sequence (see below).

[1594] T20 albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the HIV-1 inhibitory peptide T20, i.e., Tyr-643 to Phe-678. In one embodiment of the invention, T20 albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a

further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature T20 albumin fusion protein is secreted directly into the culture medium. T20 albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, T20 albumin fusion proteins of the invention comprise the native HIV-1 transmembrane protein gp41 signal sequence. In further preferred embodiments, the T20 albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2672.

Expression in yeast S. cerevisiae.

[1595] Construct 2672 can be transformed into yeast *S. cerevisiae* by methods known in the art (see Example 3). Expression levels can be examined by immunoblot detection with anti-HSA serum as the primary antibody.

Purification from yeast S. cerevisiae cell supernatant.

[1596] The cell supernatant containing the secreted T20 albumin fusion protein expressed from construct ID #2672 in yeast *S. cerevisiae* can be purified as described in Example 4. N-terminal sequencing of the albumin fusion protein should result in the sequence DAHKS (SEQ ID NO:2143) which corresponds to the amino terminus of the mature form of HSA.

The activity of T20 can be assayed using an in vitro Infectivity Assay and/or a Cell-Cell Fusion Inhibition Assay.

[1597] The *in vitro* infectivity and cell-cell fusion inhibition assays are described in Wild et al., "Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection", Proc. Natl. Acad. Sci. USA, 91: 9770-9774 (1994)).

Method

[1598] High-titered virus stocks may be prepared in CEM human leukemia cells as described previously (*see* Wild, C., et al., "A synthetic peptide inhibitor of human

immunodeficiency virus replication: correlation between solution structure and viral inhibition", Proc. Natl. Acad. Sci. USA 89: 10537-10541 (1992)). Infectious titers may be estimated by end-point dilution on AA5 and CEM continuous cell-lines. Reverse transcriptase (RT) activity present in the supernatants may be taken as criteria for successful infection. The 50% tissue culture infection dose ($TCID_{50}$) may be calculated by using the formula of Reed and Muench (see Wild et al., "Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection", Proc. Natl. Acad. Sci. USA, 91: 9770-9774 (1994)). Primary HIV-1 isolates may be expanded in activated peripheral blood mononuclear cells, "PBMC", from normal donors.

[1599] The ability of the T20 albumin fusion protein to inhibit infection with prototypic cell-free virus, i.e., HIV-1_{LAI} or HIV-1_{NIHZ}, may be evaluated by incubating serial dilutions of cell-free virus with AA5 or CEM target cells containing various concentrations of the T20 albumin fusion protein. The T20 albumin fusion protein may be tested against primary isolates and the prototypic HIV-1_{LAI} isolate in a similar assay using PBMC as target cells. Both assays are carried out as described in Wild et al., 1992.

[1600] The ability of the T20 albumin fusion protein to block virus-mediated cell-cell fusion may be assessed as described previously in Wild et al., 1992. Briefly, approximately 7 x 10^4 MOLT-4 cells may be incubated with 10^4 CEM cells and chronically infected with the HIV-1 isolates in 96-well plates (half-area cluster plates; Costar) in 100 μL of culture medium. The T20 albumin fusion protein may be added in 10 μL and the cell mixtures may be incubated for 24 hrs at 37°C. At that time, multinucleated giant cells may be estimated by microscopic examination at x40 magnification.

The activity of T20 albumin fusion encoded by construct ID # 2672 can be assayed using an in vitro Infectivity Assay and/or a Cell-Cell Fusion Inhibition Assay.

Method

[1601] The T20 albumin fusion protein encoded by construct 2672 can be tested in the *in vitro* infectivity bioassay as well as the cell-cell fusion inhibition assay as described above under subsection heading, "The activity of T20 can be assayed using an *in vitro* Infectivity Assay and/or a Cell-Cell Fusion Inhibition Assay".

EXAMPLE 109: Construct ID 2673, T20-HSA, Generation.

[1602] Construct ID 2673, pSAC35:T20.HSA, comprises DNA encoding a T20 albumin fusion protein which has the HSA chimeric leader sequence, i.e., the HSA-kex2 signal peptide, followed by the HIV-1 inhibitory peptide T20, i.e., Y643-F678, fused to the amino-terminus of the mature form of HSA in the yeast *S. cerevisiae* expression vector pSAC35.

Cloning of T20 cDNA

[1603] The DNA encoding the HIV-1 inhibitory peptide was PCR generated using four overlapping primers. The sequence was codon optimized for expression in yeast *S. cerevisiae*. The PCR fragment was digested with *Sal* I/*Cla* I and subcloned into *Xho* I/*Cla* I digested pScCHSA. A *Not* I fragment was then subcloned into the pSAC35 plasmid. Construct ID #2673 encodes for the chimeric leader sequence of HSA fused to the HIV-1 inhibitory peptide T20, i.e., Tyr-643 to Phe-678, followed by the mature form of HSA.

[1604] The 5' and 3' primers of the four overlapping oligonucleotides suitable for PCR amplification of the polynucleotide encoding the HIV-1 inhibitory peptide T20, T20-5 and T20-6, were synthesized:

T20-5: 5'-AGGAGCGTCGACAAAAGA**TACACTAGTTTGATTCATAGTTTG**-3'

(SEQ ID NO:1192)

T20-6: 5'-CGCGCATCGATGAGCAACCTCACTCTTGTGTGCATC**AAACCAATTCCACAAA**

CTAGCCCATTTATCC-3' (SEQ ID NO:1193)

T20-5 incorporates a *Sal* I cloning site (shown underlined), nucleotides encoding the last three amino acid residues of the HSA chimeric leader sequence, and the DNA encoding the first 8 amino acids (shown in bold) of the HIV-1 inhibitory peptide T20, i.e., Tyr-643 to Leu-650. In T20-6, the underlined sequence is a *Cla* I site; and the *Cla* I site and the DNA following it are the reverse complement of DNA encoding the first 10 amino acids of the mature HSA protein (SEQ ID NO:1038). The bolded sequence is the reverse complement of the 31 nucleotides encoding the last 10 amino acid residues Asp-669 to Phe-678 of the HIV-1 inhibitory peptide T20. The T20-2 and T20-3 oligonucleotides (as in Example 108) overlap with each other and with T20-5 and T20-6, respectively, and encode the HIV-1 inhibitory peptide T20. Using these primers, the HIV-1 inhibitory peptide T20 was generated by annealing, extension of the annealed primers, digestion with *Sal* I and *Cla* I, and subcloning into *Xho* I/*Cla* I digested pScCHSA. After the sequence was confirmed, the *Not* I fragment

containing the T20 albumin fusion expression cassette was subcloned into pSAC35 cut with *Not* I to generate construct ID 2673. Construct ID #2673 encodes an albumin fusion protein containing the chimeric leader sequence, the HIV-1 inhibitory peptide T20, and the mature form of HSA.

[1605] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing can confirm the presence of the expected T20 sequence (see below).

[1606] T20 albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the HIV-1 inhibitory peptide T20, i.e., Tyr-643 to Phe-678. In one embodiment of the invention, T20 albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature T20 albumin fusion protein is secreted directly into the culture medium. T20 albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, T20 albumin fusion proteins of the invention comprise the native HIV-1 transmembrane protein gp41 signal sequence. In further preferred embodiments, the T20 albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2673.

Expression in yeast S. cerevisiae.

[1607] Construct 2673 can be transformed into yeast *S. cerevisiae* by methods known in the art (see Example 3). Expression levels can be examined by immunoblot detection with anti-HSA serum as the primary antibody.

Purification from yeast S. cerevisiae cell supernatant.

[1608] The cell supernatant containing the secreted T20 albumin fusion protein expressed from construct ID #2673 in yeast *S. cerevisiae* can be purified as described in Example 4. N-terminal sequencing of the expressed and purified albumin fusion protein

should generate YTSLI (SEQ ID NO:2151) which corresponds to the amino terminus of the HIV-1 inhibitory peptide T20.

The activity of T20 albumin fusion encoded by construct ID # 2673 can be assayed using an in vitro Infectivity Assay and/or a Cell-Cell Fusion Inhibition Assay.

Method

[1609] The T20 albumin fusion protein encoded by construct 2673 can be tested in the *in vitro* infectivity bioassay as well as the cell-cell fusion inhibition assay as described above in Example 108 under subsection heading, "The activity of T20 can be assayed using an *in vitro* Infectivity Assay and/or a Cell-Cell Fusion Inhibition Assay".

EXAMPLE 110: Indications for T20 albumin fusion proteins

[1610] Based on the activity of T20 albumin fusion proteins in the above assays, T20 albumin fusion proteins are useful in treating, preventing, and/or diagnosing HIV, AIDS, and/or SIV (simian immunodeficiency virus) infections.

EXAMPLE 111: Construct ID 2667, HSA-T1249, Generation.

[1611] Construct ID 2667, pSAC35:HSA.T1249, comprises DNA encoding a T1249 albumin fusion protein which has the full length HSA protein, including the native HSA leader sequence, fused to the amino-terminus of the second-generation fusion inhibitor peptide, "T1249", i.e., W1-F39, in the yeast *S. cerevisiae* expression vector pSAC35. The T1249 peptide is a second-generation fusion inhibitor derived from the HIV-1 transmembrane protein gp41 and is shown to have inhibitory activity on HIV-1 infection.

Cloning of T1249 cDNA

[1612] The polynucleotide encoding T1249 was PCR generated using four overlapping primers T1249-1, T1249-2, T1249-3, and T1249-4, described below. The sequence was codon optimized for expression in yeast *S. cerevisiae*. The PCR fragment was cut with *Bsu* 36I/*Asc* I, and ligated into *Bsu* 36I/*Asc* I cut pScNHSA. A *Not* I fragment was then subcloned into the pSAC35 plasmid. Construct ID #2667 encodes an albumin fusion protein containing the full length HSA protein, including the native HSA leader sequence, fused to the T1249 peptide, i.e., Trp-1 to Phe-39.

[1613] The 5' and 3' primers of the four overlapping oligonucleotides suitable for PCR amplification of the polynucleotide encoding the T1249 peptide, T1249-1 and T1249-4, were synthesized:

T1249-1: 5'-AAGCTGCCTTAGGCTTA**TGGCAAGAATGGGAACAAAAG**-3'

(SEQ ID NO:1181)

T1249-2: 5'- **TGGCAAGAATGGGAACAAAAGATTACTGCTTTGTTAGAACAAGCTCAAATT CAACAAGAAAAGAATGAAT**-3' (SEQ ID NO:1206)

T1249-3: 5'- **GAACCATTCCCATAAAGAAGCCCATTTATCCAACTTTTGCAATTCATATTCA TTCTTTTCTTGTTGAATTTGAGCTT**-3' (SEQ ID NO:1207)

T1249-4: 5'-TTGGCGCGCC**TTAGAACCATTCCCATAAAGAAGCCCATTTATC**-3'

(SEQ ID NO:1182)

[1614] T1249-1 incorporates the *Bsu* 36I cloning site (shown underlined) and nucleotides encoding the last four amino acid residues of the mature form of HSA (SEQ ID NO:1038), as well as 21 nucleotides (shown in bold) encoding the first 7 amino acid residues of the T1249 peptide, i.e., Trp-1 to Lys-7. In T1249-4, the *Asc* I site is underlined and the last 30 nucleotides (shown in bold) are the reverse complement of DNA encoding the last 10 amino acid residues of the T1249 peptide, Asp-30 to Phe-39. The T1249-2 and T1249-3 oligonucleotides overlap with each other and with T1249-1 and T1249-4, respectively, and encode the T1249 peptide. The PCR product was purified (for example, using Wizard PCR Preps DNA Purification System (Promega Corp)) and then digested with *Bsu36*I and *AscI*. After further purification of the *Bsu36*I-*AscI* fragment by gel electrophoresis, the product was cloned into *Bsu36I*/*AscI* digested pScNHSA. After the sequence was confirmed, the expression cassette encoding this T1249 albumin fusion protein was subcloned into pSAC35 as a *Not* I fragment. A *Not* I fragment was further subcloned into pSAC35 to give construct ID # 2667.

[1615] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing can confirm the presence of the expected HSA sequence (see below).

[1616] T1249 albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the HIV-1 inhibitory peptide T1249, i.e., Trp-1 to Phe-39. In one embodiment of the invention, T1249 albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for

expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature T1249 albumin fusion protein is secreted directly into the culture medium. T1249 albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, T1249 albumin fusion proteins of the invention comprise the native HIV-1 transmembrane protein gp41 signal sequence. In further preferred embodiments, the T1249 albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

Expression and Purification of Construct ID 2667.

Expression in yeast S. cerevisiae.

[1617] Construct 2667 can be transformed into yeast *S. cerevisiae* by methods known in the art (see Example 3). Expression levels can be examined by immunoblot detection with anti-HSA serum as the primary antibody.

Purification from yeast S. cerevisiae cell supernatant.

[1618] The cell supernatant containing the secreted T1249 albumin fusion protein expressed from construct ID #2667 in yeast *S. cerevisiae* can be purified as described in Example 4. N-terminal sequencing of the albumin fusion protein should result in the sequence DAHKS which corresponds to the amino terminus of the mature form of HSA.

The activity of T1249 albumin fusion encoded by construct ID # 2667 can be assayed using an in vitro Infectivity Assay and/or a Cell-Cell Fusion Inhibition Assay.

Method

[1619] The T1249 albumin fusion protein encoded by construct 2667 can be tested in the *in vitro* infectivity bioassay as well as the cell-cell fusion inhibition assay as described above in Example 108 under subsection heading, "The activity of T20 can be assayed using an *in vitro* Infectivity Assay and/or a Cell-Cell Fusion Inhibition Assay".

EXAMPLE 112: Construct ID 2670, T1249-HSA, Generation.

[1620] Construct ID 2670, pSAC35:T1249.HSA, comprises DNA encoding a T1249 albumin fusion protein which has the HSA chimeric leader sequence, i.e., the HSA-kex2 signal peptide, the second-generation fusion inhibitor peptide, "T1249", i.e., W1-F39 fused to the amino-terminus of the mature form of HSA in the yeast *S. cerevisiae* expression vector pSAC35.

Cloning of T1249 cDNA

[1621] The DNA encoding the second-generation fusion inhibitor peptide was PCR generated using four overlapping primers. The sequence was codon optimized for expression in yeast *S. cerevisiae*. The PCR fragment was digested with *Sal I/Cla* I and subcloned into *Xho I/Cla* I digested pScCHSA. A *Not* I fragment was then subcloned into the pSAC35 plasmid. Construct ID #2670 encodes for the chimeric leader sequence of HSA fused to the T1249 peptide, i.e., Trp-1 to Phe-39, followed by the mature form of HSA.

[1622] The 5' and 3' primers of the four overlapping oligonucleotides suitable for PCR amplification of the polynucleotide encoding the T1249 peptide, T1249-5 and T1249-6, were synthesized:

T1249-5: 5'-AGGAGCGTCGACAAAAGA**TGGCAAGAATGGGAACAAAAG**-3'

(SEQ ID NO:1184)

T1249-6: 5'-ATCGATGAGCAACCTCACTCTTGTGTGCATC**GAACCATTCCCATAAAG**

AAGCCCATTTATC-3' (SEQ ID NO:1185)

[1623] T1249-5 incorporates a *Sal* I cloning site (shown underlined), nucleotides encoding the last three amino acid residues of the HSA chimeric leader sequence, and the DNA encoding the first 7 amino acids (shown in bold) of the T1249 peptide, i.e., Trp-1 to Lys-7. In T1249-6, the underlined sequence is a *Cla* I site; and the *Cla* I site and the DNA following it are the reverse complement of DNA encoding the first 10 amino acids of the mature HSA protein (SEQ ID NO:1038). The bolded sequence is the reverse complement of the 30 nucleotides encoding the last 10 amino acid residues Asp-30 to Phe-39 of the T1249 peptide. The T1249-2 and T1249-3 oligonucleotides (as in Example 111) overlap with each other and with T1249-5 and T1249-6, respectively, and encode the T1249 peptide. Using these primers, the T1249 peptide was generated by annealing, extension of the annealed primers, digestion with *Sal* I and *Cla* I, and subcloning into *Xho I/Cla* I digested pScCHSA. After the sequence was confirmed, the *Not* I fragment containing the T1249 albumin fusion

expression cassette was subcloned into pSAC35 cut with *Not* I to generate construct ID 2670. Construct ID #2670 encodes an albumin fusion protein containing the chimeric leader sequence, the T1249 peptide, and the mature form of HSA.

[1624] Further, analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing can confirm the presence of the expected T1249 sequence (see below).

[1625] T1249 albumin fusion proteins of the invention preferably comprise the mature form of HSA , i.e., Asp-25 to Leu-609, fused to either the N- or C- terminus of the T1249 peptide, i.e., Trp-1 to Phe-39. In one embodiment of the invention, T1249 albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature T1249 albumin fusion protein is secreted directly into the culture medium. T1249 albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MAF, INV, Ig, Fibulin B, Clusterin, Insulin-Like Growth Factor Binding Protein 4, variant HSA leader sequences including, but not limited to, a chimeric HSA/MAF leader sequence, or other heterologous signal sequences known in the art. In a preferred embodiment, T1249 albumin fusion proteins of the invention comprise the native HIV-1 transmembrane protein gp41 signal sequence. In further preferred embodiments, the T1249 albumin fusion proteins of the invention further comprise an N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants, are also encompassed by the invention.

The activity of T1249 albumin fusion encoded by construct ID # 2670 can be assayed using an in vitro Infectivity Assay and/or a Cell-Cell Fusion Inhibition Assay.

Method

[1626] The T1249 albumin fusion protein encoded by construct 2670 can be tested in the *in vitro* infectivity bioassay as well as the cell-cell fusion inhibition assay as described above in Example 108 under subsection heading, "The activity of T20 can be assayed using an *in vitro* Infectivity Assay and/or a Cell-Cell Fusion Inhibition Assay".

EXAMPLE 113: Indications for T1249 albumin fusion proteins

[1627] Based on the activity of T1249 albumin fusion proteins in the above assays, T1249 albumin fusion proteins are useful in treating, preventing, and/or diagnosing HIV, AIDS, and/or SIV (simian immunodeficiency virus) infections.

EXAMPLE 114: Construct ID 2702, HSA-GCSF.T31-L201, Generation

[1628] Construct ID 2702, pSAC35:HSA.GCSF.T31-L201, comprises DNA encoding a GCSF albumin fusion protein which has mature HSA fused downstream of the HSA/kex2 leader sequence and upstream of amino acids T31 to L201 of GCSF, in the yeast *S. cerevisiae* expression vector pSAC35.

Cloning of GCSF cDNA

[1629] The polynucleotide encoding the GCSF C-terminal deletion mutant was PCR amplified using primers GCSF-5 and GCSF-6, described below. The amplimer was cut with *Bsu36*I and *Asc*I, and ligated into pScNHSA. Construct ID #2702 encodes an albumin fusion protein containing mature HSA fused downstream of the HSA/kex2 leader sequence and upstream of amino acids T31 to L201 of GCSF.

[1630] Two oligonucleotide primers, GCSF-5 and GCSF-6, suitable for PCR amplification of the polynucleotide encoding the GCSF C-terminal deletion mutant, were synthesized:

GCSF-5: 5'- AAGCTGCCTTAGGCTTAACCCCCCTGGGCCCTGCCAG (SEQ ID NO: 1197)

GCSF-6: 5'- GCGCGCGGCGCGCCTCAAAGGTGGCGTAGAACGCGGTACGAC (SEQ ID NO: 1198)

[1631] GCSF-5 incorporates the *Bsu*36I cloning site (shown underlined), and nucleotides encoding the last six amino acids of HSA as well as the first six amino acids of mature GCSF (amino acids T31 through A36). GCSF-6 contains an *Asc*I cloning site (shown underlined) and the last 25 nucleotides are the reverse complement of DNA encoding the last eight amino acid residues of the GCSF C-terminal deletion mutant (S194 through L201). The PCR product generated with these primers was purified (for example, using Wizard PCR Preps DNA Purification System (Promega Corporation)) and then digested with *Bsu*36I and *Asc*I. After further purification of the *Bsu*36I/*Asc*I PCR fragment by gel elelctrophoresis, the product was cloned into *Bsu*36I/*Asc*I digested pScNHSA. After the sequence was confirmed,

the expression cassette encoding this GCSF albumin fusion protein was subcloned into pSAC35 as a *Not*I fragment.

[1632] Further analysis of the N-terminus of the expressed albumin fusion protein by amino acid sequencing can confirm the presence of the expected HSA sequence (see below).

[1633] GCSF albumin fusion proteins of the invention preferably comprise the mature form of HSA, i.e., Asp-25 to Leu-609, fused to either the N- or C-terminus of the C-terminal deletion mutant of GCSF, i.e., T31 to L201. In one embodiment of the invention, GCSF albumin fusion proteins of the invention further comprise a signal sequence which directs the nascent fusion polypeptide in the secretory pathways of the host used for expression. In a further preferred embodiment, the signal peptide encoded by the signal sequence is removed, and the mature GCSF albumin fusion protein is secreted directly into the culture medium. GCSF albumin fusion proteins of the invention may comprise heterologous signal sequences including, but not limited to, MFα-1, Invertase, Ig, Fibulin B, Clusterin, Insulin-like growth factor binding protein 4, K. lactis killer toxin, and variant HSA leader sequences including, but not limited to, a chimeric HSA/MFα-1 (HSA/kex2) leader sequence, a chimeric K.lactis/MFα-1 leader sequence, or other heterologous signal sequences known in the art. In a further preferred embodiment, GCSF albumin fusion prteins of the invention comprise the native GCSF signal sequence. In further preferred embodiements, the GCSF albumin fusion proteins of the invention further comprise and N-terminal methionine residue. Polynucleotides encoding these polypeptides, including fragments and/or variants are also encompassed by the invention.

Expression and Purification of Construct ID #2702

Expression in yeast S. cerevisiae

[1634] Construct #2702 was transformed into yeast *S. cerevisiae* by methods known in the art (see Example 3) and as previously described for construct ID #1642 (see Example 19). Expression levels were examined by immunoblot detection with anti-HSA serum as the primary antibody (data not shown).

Purification from yeast S. cerevisiae cell supernatant

[1635] A general procedure for purification of albumin fusion proteins is described in Example 4. The cell supernatant containing GCSF albumin fusion protein expressed from construct ID #2702 in yeast *S. cerevisiae* was purified as described in Example 20. N-

terminal sequencing of the albumin fusion protein should result in the sequence DAHKS which corresponds to the amino terminus of the mature form of HSA.

The activity of GCSF albumin fusion encoded by construct ID # 2702 can be assayed using an in vitro NFS-60 cell proliferation assay.

Method

[1636] The GCSF albumin fusion protein encoded by construct 2702 was tested using the *in vitro* NFS-60 cell proliferation bioassay previously described in Example 19 under subsection headings "The activity of GCSF can be assayed using an in vitro NFS-60 cell proliferation assay" and "The activity of GCSF albumin fusion encoded by construct ID # 1642 can be assayed using an in vitro NFS-60 cell proliferation assay".

Results

[1637] Both the partially purified GCSF albumin fusion protein encoded by construct 1634 (HSA-GCSF) and the GCSF C-terminal deletion mutant albumin fusion protein (L-171) encoded by construct 2702 demonstrated the ability to cause NFS-60 cell proliferation, with the C-terminal deletion mutant exhibiting a more potent proliferative effect (see Figure 19). Unexpectedly, the fusion protein encoded by construct 2702 exhibited 2-3 times more activity than the fusion protein encoded by construct 1643. Alternate GCSF albumin fusion constructs comprise albumin fused to amino acid residues 1-169 of mature GCSF and albumin fused to amino acid residues 1-170 of mature GCSF.

EXAMPLE 115: Construct ID 2876, HSA-IFNα hybrid

[1638] Construct ID 2876, pSAC35:HSA.IFNαA(C1-Q91)/D(L93-E166) R23K,A113V comprises DNA encoding an IFNα hybrid albumin fusion protein which has mature HSA fused downstream of the HSA/kex2 leader sequence and upstream of an IFNα A/D hybrid amino acid sequence, in the yeast S. cerevisiae expression vector pSAC35. Regarding the composition of the hybrid IFN, the first 91 amino acids are from the subtype IFNα2 (also called IFNαA) and the remaining 75 aa are from IFNα1 (IFNαD). We incorporated two point mutations (R23K, A113V). The fusion was generated by PCR and fused downstream of HSA within the yeast expression vector pSAC35.

Results

CID 2876 Expression and Purification

[1639] The yeast strain BXP-10 was transformed with pSAC35:CID 2876 and a transformant selected for fermentation. A 5-liter fermentation was performed and analysis of supernatant demonstrated high expression (approximately 500 mg/l). A small proportion of the supernatant was processed to pilot purification. Approximately 1 mg of CID 2876 protein (greater than 95% pure based on N-terminal sequence) was obtained following a purification through Blue-sepharose, followed by gel filtration, followed by Q-anion exchange. The remaining fermentation starting material is available for further purification if needed.

ISRE Activity

[1640] All type I IFNs mediate their activities through engagement of a common IFN receptor complex and activation of the ISRE signal transduction pathway. Activation of gene transcription through this pathway leads to the cellular responses associated with IFNs including anti-proliferation, antiviral and immune modulation. Using a reporter based strategy, the ability of CID 2876 to activate the ISRE signal transduction pathway was determined. CID 2876 was found to be a potent activator of the ISRE pathway, demonstrating an EC_{50} of 2.7 ng/ml (data not shown). This compares favorably with the potency of CID 3165 in this assay system.

Anti-viral activity

[1641] A hallmark activity of IFNs is their ability to mediate cellular protection against viral infection. While most human type I IFNs display antiviral activity in a species restricted manner, the hybrid IFN employed in this study has been demonstrated to be active on murine cells. Thus the antiviral activity of CID 2876 was evaluated on the murine cell line L929 infected with EMCV. Results indicate that CID 2876 does demonstrate antiviral activity in a cross species manner (data not shown).

Example 116: Activity of Construct 3070 (GLP-1 Albumin Fusion) Measured by *In Vitro* Stimulation of Insulin mRNA in INS-1 Cells

[1642] It has recently been shown that GLP-1 increases the expression of insulin mRNA in pancreatic beta-cells (Buteau et al., Diabetologia 1999 Jul;42(7):856-64). Thus, the ability of the GLP-1 albumin fusion protein encoded by CID 3070 to stimulate insulin mRNA was evaluated using the pancreatic beta-cell line INS-1 (832/13).

[1643] Figure 14 illustrates the steady-state levels of insulin mRNA in INS-1 (832/13) cells after treatment with GLP-1 or GLP-1 albumin fusion protein encoded by construct ID 3070 (CID 3070 protein). Both GLP-1 and the CID 3070 protein stimulate transcription of the insulin gene. The first bar (black) represents the untreated cells. Bars 2-4 (white) represent cells treated with the indicated concentrations of GLP-1. Bars 5-7 (gray) represent cells treated with the indicated concentrations of CID 3070 protein.

[1644] It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

[1645] The entire disclosure of each document cited (including patents, patent applications, patent publications, journal articles, abstracts, laboratory manuals, books, or other disclosures) as well as information available through Identifiers specific to databases such as GenBank, GeneSeq, or the CAS Registry, referred to in this application are herein incorporated by reference in their entirety.

[1646] Furthermore, the specification and sequence listing of each of the following U.S. applications are herein incorporated by reference in their entirety: U.S. Application No. 60/341,811, filed December 21, 2001; U.S. Application No. 60/360,000, filed February 28, 2002; U.S. Application No. 60/378,950, filed May 10, 2002; U.S. Application No. 60/398,008, filed July 24, 2002; U.S. Application No. 60/411,355, filed September 18, 2002; U.S. Application No. 60/414,984, filed October 2, 2002; U.S. Application No. 60/417,611, filed October 11, 2002; U.S. Application No. 60/420,246, filed October 23, 2002; U.S. Application No. 60/423,623, filed November 5, 2002; U.S. Application No. 60/350,358, filed January 24, 2002; U.S. Application No. 60/359,370, filed February 26, 2002; U.S. Application No. 60/367,500, filed March 27, 2002; U.S. Application No. 60/402,131, filed August 9, 2002; U.S. Application No. 60/402,708, filed August 13, 2002; U.S. Application No. 60/351,360, filed January 28, 2002; U.S. Application No. 60/382,617, filed May 24, 2002; U.S. Application No. 60/383,123, filed May 28, 2002; U.S. Application No. 60/385,708, filed June 5, 2002; U.S. Application No. 60/394,625, filed July 10, 2002; U.S. Application No. 60/411,426, filed September 18, 2002; U.S. Application No. 60/370,227, filed April 8, 2002; International Application No. PCT/US02/40891, filed December 23, 2002; and International Application No. PCT/US02/40892, filed December 23, 2002.

Furthermore, the specification and sequence listing of U.S. application, Human Genome Sciences, Inc. attorney docket number PF574, filed concurrently herewith on February 11, 2004, is hereby incorporated by reference in its entirety.